Hydrolysis of Steroid Conjugates

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The metabolites of the steroid hormones appear in the urine almost exclusively as water soluble conjugates. Compounds of the 3β-Δ^5- or 5α-series are mainly excreted as sulphates, while the remainder are excreted as β-glycosidically linked glucuronides. However, there are exceptions: for example, 80% of the androsterone is excreted as the sulphate, oestriol as the glucuronide, and oestrone and oestradiol also as the sulphate [1]. The mechanism of the formation of the sulphate [2] and glucuronide [3] can be considered solved. Whether other types of conjugate exist is an open question. Recent work indicates that they may [4,5].

In order to determine steroids they must first be converted to the free alcohols. The enzymatic hydrolysis of steroids is becoming increasingly important because of its specificity and simplicity.

Principle

Steroid hydrolases (β-glucuronidase, phosphatases, sulphatases) catalyse the hydrolysis of the steroid conjugate to the steroid alcohol and the free acid:

(1) $$\text{Conjugate} + H_2O \rightleftharpoons \text{steroid alcohol} + \text{acid}$$

Under the conditions described here the equilibrium of the reaction lies far to the right. With biological material the steroid alcohol is determined, but with pure solutions and extracts the acid liberated can also be estimated.

This survey consists of the following sections:

A. Hydrolysis of steroid glucuronides
B. Hydrolysis of steroid sulphates
C. Hydrolysis of steroid phosphates
D. Hydrolysis of steroid conjugates and the determination of the acid liberated
   I. Glucuronide
   II. Sulphate
   III. Phosphate

A. Hydrolysis of Steroid Glucuronides

Since the discovery of β-glucuronidase by *Cohen* and *Marrian* [6], and by *Patterson* [7] the enzymatic hydrolysis of steroid glucuronides has been widely used. β-Glucuronidase preparations have been obtained from mammalian liver and spleen, and from *Escherichia coli*. These preparations differ in their pH optimum and also in their substrate specificity. In spite of this, the considerable degree of agreement in the methods for the enzymatic hydrolysis allows a single general description.

Reagents

1. Sodium acetate · 3 H_2O, A. R.
2. Acetic acid, glacial
3. Tris-hydroxymethyl-aminomethane, tris

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[1] Review: *J. Tamm* and *K. D. Voigt*, Acta endocrinol. Suppl. *54*, 1 [1960].
[2] *Y. Nose* and *F. Lipmann*, J. biol. Chemistry *233*, 1348 [1958].
[3] *K. J. Isselbacher*, Recent Prog. Hormone Res. *12*, 134 [1956].
[4] *G. W. Oertel* and *K. Eik-Nes*, Acta endocrinol. *30*, 93 [1959].
[5] *K. D. Voigt* and *W. Schroeder*, Acta endocrinol. *21*, 343 [1956].
[6] *S. L. Cohen* and *G. F. Marrian*, Biochem. J. *29*, 1577 [1935].
[7] *J. Patterson*, Brit. med. J. *2*, 522 [1937].

4. Ether, A. R.
5. Ethanol, 96%
6. Cellosolve B (b. p. 68—73°C)
7. Sodium hydroxide, 0.1 N, A. R.
8. β-Glucuronidase
   from ox liver or bacteria. Commercial preparation, see p. 975.

Purity of the enzyme preparation

The activity of the β-glucuronidase is given[8] in *Fishman* units*). Commercial preparations vary between 100 and 1000 units/mg. protein. The preparation should not be contaminated with more than 0.01% of sulphatase or phosphatase (relative to the specific activity of the glucuronidase), since the presence of these enzymes can lead to erroneous results in the analysis of unknown steroid conjugates. For further properties of β-glucuronidase see the section on "Specificity".

Preparation of Solutions

Prepare the buffer solutions and their dilutions with fresh, doubly distilled water. Because of the danger of bacterial contamination strict conditions of cleanliness should be observed.

I. Acetate buffer (0.1 M; pH 4.62)**):
   a) Dissolve 13.61 g. Na acetate·3 H_2O in doubly distilled water and make up to 1000 ml.
   b) Dilute 6.00 g. acetic acid to 1000 ml. with doubly distilled water.

   Mix equal parts of solutions a) and b)

II. Tris buffer (0.3 M; pH 6.5):
   Dissolve 36.3 g. tris-hydroxymethyl-aminomethane in 100 ml. doubly distilled water and adjust to pH 6.5 by the dropwise addition of glacial acetic acid. Dilute to 1000 ml. with doubly distilled water.

III. β-Glucuronidase (250 units/ml.):
   Dilute or dissolve commercial preparations with acetate buffer (solution I) or (Sigma glucuronidase) with tris buffer (solution II).

Stability of the solutions

Store the enzyme solution, stoppered, at 4°C. It keeps for 3—4 weeks. For determinations on blood prepare the glucuronidase solution freshly for each series. Do not use the solutions if they are contaminated with bacteria or moulds.

Procedure

Experimental material

Work up urine as quickly as possible. During the period of the collection of the urine the patients should receive no medication and they should be confined to bed. For the determination of steroids in plasma 60—80 ml. blood are required. Moisten the walls of the

*) 1 *Fishman* unit is the amount of enzyme which liberates 1 μg. phenolphthalein from phenolphthalein glucuronide in 1 hour at 37°C.

**) The buffer is available commercially as a standard acetate solution pH 4.62 (from E. Merck, Darmstadt, Germany).

8) *P. Talalay, W. H. Fishman* and *C. Huggins*, J. biol. Chemistry *166*, 757 [1946].

syringe and the container with 0.5 ml. heparin solution (Liquemin "Roche", 5000 units). Centrifuge off the formed elements not later than 30 min. after the collection of the blood. The steroid content of the clear, haemolysis-free plasma does not change when stored for a day in a cold room at 4° C. If the analysis cannot be carried out for several days store the plasma at −16 to −20° C.

Extraction and deproteinization

Urine: Boil for a short time to inactivate enzyme inhibitors.

Plasma: If only the free or total steroid content is to be determined, then heating at 60° C for 30 min. is sufficient. For the separate determination of free and conjugated steroids, the free steroids must be extracted and then the plasma must be deproteinized. The preliminary treatment is the same, whether sulphates, glucuronides, phosphates or C_{21} and C_{19} steroids are to be determined.

Remove free steroids from the plasma by extracting twice with three volumes of chloroform *). The aqueous phase retains the steroid conjugates. To deproteinize add five volumes of 96% ethanol, mix and heat to 40° C in a water bath. Allow to stand for 24 hours at 4° C and filter the supernatant into a round-bottomed flask. Wash the precipitate with two 50 ml. portions of 96% ethanol and centrifuge. Combine the ethanolic extracts and distill off the ethanol *in vacuo*. Extract the aqueous residue with two 100 ml. portions of ether to remove lipides. Discard the ether and free the aqueous phase from dissolved ether by heating on a water bath (not over 50° C). Add ethanol to a final concentration of 70% and wash with two 100 ml. portions of Cellosolve B (saturated with 70% ethanol). Discard the Cellosolve phase. Remove the ethanol *in vacuo*. The residual solution should be practically free from ethanol. Concentration to 4−5 ml. is usually sufficient.

Enzymatic hydrolysis

1. *Urine:* In a stoppered 50 ml. centrifuge tube add

16 ml. glucuronidase solution (III)

to

4 ml. fresh (boiled) urine.

Incubate for 48 hours at 37° C (incubator). Extract the steroid alcohols liberated with two

25 ml. portions of chloroform

in the centrifuge tube. Suck off the chloroform with a 50 ml. syringe which has a long, blunt steel cannula. Wash the combined chloroform phases with

4 ml. 0.1 N NaOH,

discard the aqueous phase and use the chloroform phase for the steroid determination.

2. *Plasma:* For 100 ml. plasma 62500 units of β-glucuronidase are required. As 60−80 ml. blood yield 25−36 ml. plasma, 16625−21875 units are required for each analysis.

In stoppered centrifuge tubes dilute

4 ml. deproteinized extract

with

12 ml. acetate buffer (soln. I) or tris buffer (soln. II).

*) If complete extraction of C_{21} and C_{19} steroids is required, extract C_{21} steroids with ethyl acetate (twice) and C_{19} steroids with benzene (three volumes each time).

Mix by inversion, add

67 ml. β-glucuronidase solution (III)

and incubate for 48 hours at 37° C (incubator). Extract the steroid alcohols liberated with two

150 ml. portions of ether (peroxide-free)

in a separating funnel. Wash the combined ether phases with three

5 ml. portions of 0.1 N NaOH

and then with three

10 ml. portions of doubly distilled water.

Concentrate the washed ether phase to dryness on a steam bath. Use the residue for the steroid determination.

Sources of Error

If free and conjugated steroids are to be determined quantitatively, then the biological material must be worked up rapidly. Hydrolysis of conjugates by endogenous (plasma glucuronidase) and exogenous (bacterial contamination) factors, and loss of steroids by adsorption on the erythrocytes can lead to false values.

Treatment of the patient with drugs or the addition of preservatives to blood or urine can give rise to complications (enzyme inhibitors, interference with the steroid determination).

Specificity

So far little work has been done on the question of whether β-glucuronidases from different sources hydrolyse all steroid glucuronides to the same extent. Certain findings[10] lead to the following conclusions:

1. The configuration of the OH group at C-3 is not important for the hydrolytic action of the β-glucuronidase from ox liver. On the other hand, the position of the OH group, to which glucuronic acid is bound, on the ring skeleton or on the side chain is important for the action of the enzyme.

2. Glucuronidases from ox liver and bacteria hydrolyse pure steroid glucuronides at different rates. Incubation of urine with both enzymes gives different results for the amounts of steroid and glucuronic acid liberated.

3. The activity of glucuronidases with phenolphthalein glucuronide does not always agree with their activity with steroid glucuronides. It is therefore always necessary to work with a large excess of enzyme and to use a longer incubation time than the activity with phenolphthalein glucuronide indicates.

B. Hydrolysis of Steroid Sulphates

The hydrolysis of steroid sulphates by the steroid sulphatase from the digestive juice of molluscs[11] and from mammalian liver[12] has not so far been used to any great extent. The enzyme is a little difficult to handle (it has not been possible to obtain a soluble enzyme preparation) and it has a high steric specificity. It only hydrolyses 3β-sulphates of the Δ^5- and 5α-series, and oestrogen sulphate. The possibility of specifically estimating the levels of the sulphate ester of Δ^5-androstene-3β-ol-17-one (dehydroisoandrosterone), which are of clinical interest in several endocrine diseases, has so far hardly been exploited.

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9) *J. Tamm, I. Beckmann* and *K. D. Voigt*, Acta endocrinol. *27*, 403 [1958].
10) *K. D. Voigt, M. Lemmer* and *J. Tamm*, Biochem. Z. *332*, 550 [1960].
11) *P. Jarridge* and *R. Henry*, Bull. Soc. chim. biol. *34*, 265 [1956].
12) *H. Gibian* and *G. Bratfisch*, Hoppe-Seylers Z. physiol. Chem. *305*, 265 [1956].

Reagents

1. Sulphuric acid, A. R., 4 N
2. n-Butanol
3. Triethanolamine hydrochloride
4. Acetic acid, glacial, A. R.
5. Ethyl alcohol, 96% (w/v)
6. Steroid sulphatase
   dry powder from liver. Commercial preparation, see p. 975.

Purity of the enzyme preparation

A unit is the amount of enzyme which, with continual shaking, liberates 1 µg. Δ^5-androstene-3β-ol-17-one from the sulphate ester in 1 hour at 37° C[13]. Contamination of the preparation with β-glucuronidase or phosphatase, which can lead to false results, should not exceed 1% (relative to the specific activity of the sulphatase).

Preparation of Solutions

I. Triethanolamine buffer (0.5 M; pH 7.3):
Dissolve 92.5 g. triethanolamine hydrochloride in 500 ml. distilled water, adjust to pH 7.3 with acetic acid and dilute to 1000 ml. with distilled water.

II. Steroid sulphatase (200 mg. protein/ml. = 200 units/ml.):
With vigorous stirring, suspend 2 g. steroid sulphatase in buffer (solution I) and make up to 10 ml. Just before use shake the enzyme suspension.

Stability of the solutions

The enzyme suspension keeps for 3—4 weeks at 4° C. The buffer is usable indefinitely, providing it is not contaminated with micro-organisms.

Procedure

Experimental material

See Section A, p. 463.

Extraction and deproteinization

Urine: Concentrate a 24 hour urine specimen to 400—500 ml. *in vacuo* and adjust the pH to between 2.5 and 3.0 (indicator paper) with 4 N H_2SO_4. Extract for 16 hours in a *Kutscher-Steudel* apparatus with 0.5 volumes of n-butanol*).

Wash the butanol extract four times with $^1/_{10}$ of its volume of distilled water containing a little sodium chloride. Distill off the butanol *in vacuo* (temperature <50° C).

Plasma: see Section A, p. 464.

Enzymatic hydrolysis

Dissolve the extract from plasma or urine in

100—200 ml. triethanolamine buffer (I),

add 1—2.5 ml. steroid sulphatase suspension (II) (200—500 units).

*) The steroid conjugates can also be extracted according to *Kellie* and *Wade*[14]: add ammonium sulphate to the urine to 50% saturation and extract three times with 2 volumes ether-ethanol (3:1). Concentrate the ether-ethanol extract to dryness and purify the residue by partition between Cellosolve B and 70% alcohol (see Section A, p. 464).

13) *H. Langecker*, Acta endocrinol. *23*, 72 [1956].

14) *A. E. Kellie* and *A. P. Wade*, Biochem. J. *66*, 196 [1957].

Incubate for 17 hours at 37°C (incubator) with continuous stirring so that the enzyme does not sediment. Add

300–600 ml. ethanol,

centrifuge for 15 min. at 1000–2000 g and wash the sediment three times with

50 ml. portions of ethanol.

Combine the supernatant and the washings and concentrate to dryness *in vacuo*. Use the residue for the steroid determination.

Specificity

Steroid sulphatase has a high steric specificity[15–17] (see the introduction to this Section, p. 465). In this respect it is different from the arylsulphatase detected in liver microsomes[18]. This specificity restricts the use of the steroid sulphatase in the hydrolysis of steroid sulphates from biological material.

C. Hydrolysis of Steroid Phosphates

Steroid phosphates have recently been demonstrated in human blood[4]. For the description of their hydrolysis, see Section D III, p. 473.

D. Hydrolysis of Steroid Conjugates and Determination of the Acid Liberated

I. Glucuronide

Principle

The following method is based on the colour reaction of *Fishman* and *Green*[20]. Glucuronic acid esters, glucuronides, free glucuronic acid and other aldehydes, especially hexoses, react with naphthoresorcinol (1,3-dihydroxynaphthalene) to give a blue colour. After treatment of the sample with hypoiodite at alkaline pH (oxidation of all free aldehydes), *only glucuronides still give the colour reaction*. The colour intensity of an oxidized sample not treated with β-glucuronidase is measured against a sample which is oxidized after enzymatic hydrolysis. The difference in intensity corresponds to the steroid glucuronide content of the sample, providing that the sample does not contain other glucuronides which may be hydrolysed by the enzyme.

Reagents

1. Sodium acetate, A. R., anhydrous
2. Acetic acid, glacial, A. R.
3. Sodium hydrogen carbonate, $NaHCO_3$, A. R.
4. Sodium carbonate, A. R., anhydrous
5. Iodine, sublimed
6. Potassium iodide, A. R.
7. Sodium bisulphite, A. R., $Na_2S_2O_5$
8. 1,3-Dihydroxynaphthalene (naphthoresorcinol)
9. Sulphuric acid, A. R., conc.

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15) *A. B. Roy*, Biochem. J. *66*, 700 [1957].
16) *S. K. Stitch* and *I. D. K. Halkerston*, J. Endocrinol. *9*, 36 [1953].
17) *K. D. Voigt*, *M. Lemmer* and *J. Tamm*, Biochem. Z. *331*, 356 [1959].
18) *K. S. Dodgson* and *B. Spencer*, Biochem. J. *53*, 444 [1953].

10. Toluene, A. R.
11. Ethanol, 95% (w/v)
12. Phenolphthalein glucuronide or 8-hydroxyquinoline glucuronide *).
13. β-Glucuronidase
    from liver or bacteria. Commercial preparation, see p. 975

Purity of the enzyme preparation
See Section A, p. 463.

Preparation of Solutions

I. Acetate buffer (0.1 M; pH 4.62) **):
   see p. 463.

II. Carbonate buffer (0.44 M; pH 10.1):
   Dissolve 8.4 g. $NaHCO_3$ and 36.0 g. Na_2CO_3 in doubly distilled water and make up to 1000 ml.

III. Iodine-KI solution:
   Dissolve 1.666 g. KI in doubly distilled water and, while warming carefully, introduce 1.291 g. iodine. After complete solution, dilute to 100 ml. with doubly distilled water.

IV. Sulphuric acid (18 N):
   Carefully pour 1 volume conc. H_2SO_4 in a thin stream into 1 volume doubly distilled water.

V. Sulphuric acid (6 N):
   Dilute solution IV three-fold with doubly distilled water.

VI. Naphthoresorcinol solution (0.4% w/v):
   Powder the naphthoresorcinol finely in a mortar. Suspend 400 mg. in 100 ml. doubly distilled water, shake vigorously for 10 min. and filter. Store the solution in a dark bottle.

VII. Sodium bisulphite (1 M):
   Dissolve 19.01 g. $Na_2S_2O_5$ in doubly distilled water and make up to 100 ml.

VIII. Glucuronide standard solution (10^{-2} M):
   Dissolve 51.6 mg. phenolphthalein glucuronide or 8.6 mg. 8-hydroxyquinoline glucuronide in doubly distilled water and make up to 10 ml.

IX. β-Glucuronidase (1000 units ***)/ml.):
   Dilute or dissolve the commercial preparation in acetate buffer (solution I).

Stability of the solutions
Prepare the naphthoresorcinol solution freshly for each series of measurements. Prepare the $Na_2S_2O_5$ solution freshly each week. Store the enzyme solution, stoppered, at 4° C. In this state it keeps for 3—4 weeks. All the other solutions keep practically indefinitely.

Procedure

Experimental material
Extract and deproteinize biological material (see Section A, p. 463). Further purification (by solvent partition[9] or column chromatography[19]) is frequently necessary to obtain the

*) for example, from Serva-Entwicklungslabor, Heidelberg, Germany.
**) The buffer is available commercially as a standard acetate solution pH 4.62 (from E. Merck, Darmstadt, Germany).
***) Definition of a unit, see Section A, p. 463.
19) *J. J. Schneider* and *M. L. Lewbart*, Recent Prog. Hormone Res. *15*, 201 [1959].

purest possible extracts. The presence of large amounts of non-steroid glucuronides can lead to considerable errors.

Assay

The correct functioning of the assay system is first checked with 0.1 ml. glucuronide standard solution (VIII) instead of the sample.

Enzymatic reaction and oxidation:

Pipette into three test tubes:

1. 2.5 ml. acetate buffer (solution I)
   0.2 ml. sample
   0.25 ml. β-glucuronidase solution (IX)
   doubly distilled water to 5 ml.
2. all solutions with the exception of β-glucuronidase solution (IX)
3. only 0.25 ml. β-glucuronidase solution (IX).

Mix thoroughly and incubate for 24 hours in a water bath at 37°C. Then pour the contents of tube 2 into tube 3 and immediately place tubes 1 and 3 in an ice bath. Add

2.05 ml. carbonate buffer (solution II)

and mix. Pipette into both tubes

1.50 ml. iodine-KI solution (III),

stopper and allow to stand for 30 min. in the dark. Add

0.15 ml. $Na_2S_2O_5$ solution (VII),

shake until the yellow colour disappears; if necessary, add a further drop of $Na_2S_2O_5$ solution (VII). Add

0.30 ml. 6 N H_2SO_4 (solution V)

and shake until the CO_2 evolution ceases.

Colour reaction:

Wavelength: 578 mμ; light path: 1 cm.; Measure against a blank (composition: as for standards, but containing doubly distilled water instead of the glucuronide standard solution).

Standards: Pipette into test tubes:

0.01 to 0.1 ml. (0.1 to 1.0 μmole) glucuronide standard solution (VIII)
doubly distilled water to 5 ml.
2.05 ml. carbonate buffer (solution II)
1.50 ml. iodine-KI solution (III).

Stopper and allow to stand for 30 min. in the dark. Add

0.15 ml. $Na_2S_2O_5$ solution (VII),

shake until the yellow colour disappears; if necessary, add a further drop of $Na_2S_2O_5$ solution (VII). Add

0.30 ml. 6 N H_2SO_4 (solution V)

and shake until the CO_2 evolution ceases. Proceed as for the samples.

Samples: Pipette into 50 ml. Erlenmeyer flasks with ground-glass stoppers:

4.0 ml. of the oxidized sample
2.0 ml. 18 N H_2SO_4 (solution IV)
2.0 ml. naphthoresorcinol solution (VI).

Mix thoroughly and place in a boiling water bath for 90 min. without a stopper (the level of the water in the bath should be higher than the level of the solutions in the flasks). Cool for 10 min. under running water, add

10 ml. ice-cold 95% ethanol,

shake and add

8 ml. toluene.

Stopper the tubes, shake vigorously for 40 sec., allow the phases to separate and carefully suck off the lower aqueous phase with a pipette. Pour part of the blue toluene phase into a cuvette and read the optical density at 578 mμ against the blank.

Calculations

Plot the optical densities of the standards against the μmoles glucuronide/tube. From this standard curve obtain the glucuronide content of the samples. According to our standard curves 0.390 μmoles glucuronide in the colour reaction gives an optical density of 1.0. Therefore instead of preparing a standard curve, the results can be obtained with sufficient accuracy by assuming:

$$E \times 0.39 = \mu\text{moles glucuronide taken for the colour reaction.}$$

To obtain the μmoles glucuronide/ml. sample the following formula is used:

$$(E_2 - E_1) \times 0.39 \times 5 \times 2.25 = \mu\text{moles glucuronide/ml. sample.}$$

where E_2 = optical density of the sample not hydrolysed with β-glucuronidase
E_1 = optical density of the sample hydrolysed with β-glucuronidase
5 = conversion from the 0.2 ml. sample taken for the enzymatic reaction to 1.0 ml.
2.25 = conversion from the 4 ml. (= portion of the oxidized mixture used for the colour reaction) to 9 ml. (= total volume of the oxidized mixture).

Example

0.2 ml. of fresh, boiled urine was analysed. The following values were measured:

Sample 1 (after enzymatic hydrolysis): $E_1 = 0.100$
Sample 2 (without enzymatic hydrolysis): $E_2 = 0.500$
$E_2 - E_1 = 0.400$

$0.4 \times 0.39 \times 5 \times 2.25 = 1.755$ μmoles glucuronide/ml. urine.

Sources of Error

Inhibitors of glucuronidases are saccharo-1,4-lactone[26], primary alcohols and steroids[10], and unknown factors in blood[27] and tissue. Glucuronic acid and glucuronolactone in large excess also inhibit.

Do not allow the tubes to stand in direct sunlight during the colour reaction. Concentrations of hexoses and disaccharides above 2×10^{-4} M interfere with the colour reaction.

II. Sulphate

Principle

Free sulphate is precipitated from acid-acetone solution by benzidine. The benzidine-sulphate complex gives a red colour with β-naphthoquinone sulphonic acid[21,22] and this colour is measured at 564 mμ.

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20) *W. H. Fishman* and *S. Green*, J. biol. Chemistry *215*, 527 [1955].
21) *H. Hilz*, Habilitationsschrift, Universität Hamburg (Germany) 1960.
22) *J. Lange* and *H. Tarven* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 999.
23) *K. H. Garbade* in: Methods for the "Photometer Eppendorf". Published by Netheler & Hinz GmbH, Hamburg, Germany.
24) *K. Lohmann* and *L. Jendrassik*, Biochem. Z. *178*, 419 [1926].
25) *C. H. Fiske* and *Y. Subbarow*, J. biol. Chemistry *66*, 375 [1925].
26) *G. A. Levvy*, Biochem. J. *52*, 464 [1952].
27) *W. H. Fishman, K. I. Altman* and *B. Springer*, Fed. Proc. *7*, 154 [1948].

The difference in the amount of free sulphate before and after enzymatic hydrolysis gives the amount of steroid-bound sulphate.

Reagents

1. Sodium sulphate, $Na_2SO_4 \cdot 10\ H_2O$, A. R.
2. Trichloroacetic acid
3. Acetone, A. R.
4. Benzidine
5. Borax (sodium tetraborate), $Na_2B_4O_7 \cdot 10\ H_2O$
6. Sodium hydroxide, A. R., 0.1 N
7. β-Naphthoquinone sulphonic acid
   sodium salt
8. Dehydroisoandrosterone sulphate, (Δ^5-androstene-3β-ol-17-on-3-sulphate), DHEA
9. Tris-hydroxymethyl-aminomethane, tris
10. Acetic acid, 96% (w/v)
11. Steroid sulphatase
    dry powder from liver. Commercial preparation, see p. 975.

Purity of the enzyme preparation

See Section B, p. 466.

Preparation of Solutions

I. Sulphate standard solution (10^{-2} M):
Dissolve 322.2 mg. $Na_2SO_4 \cdot 10\ H_2O$ in doubly distilled water and make up to 100 ml.

II. Trichloroacetic acid (2 N):
Dissolve 32.68 g. trichloroacetic acid in doubly distilled water and make up to 100 ml.

III. Benzidine solution (1% w/v):
Dissolve 1 g. benzidine in 100 ml. acetone.

IV. Borax solution (1% w/v):
Dissolve 1 g. $Na_2B_4O_7 \cdot 10\ H_2O$ in 100 ml. 0.1 N NaOH.

V. Naphthoquinone sulphonic acid (*ca.* 6×10^{-3} M):
Dissolve 0.15 g. sodium-β-naphthoquinone sulphonate in 100 ml. doubly distilled water.

VI. Tris-acetate buffer (1 M tris; pH 7.3):
Dissolve 242 g. tris-hydroxymethyl-aminomethane in doubly distilled water and make up to 1000 ml. Dilute 100 ml. of this solution with 30 ml. doubly distilled water and adjust to pH 7.3 (glass electrode) by the dropwise addition of 96% acetic acid. Dilute to 200 ml. with doubly distilled water.

VII. Steroid sulphate standard solution (10^{-2} M):
Dissolve 39.2 mg. dehydroisoandrosterone sulphate in doubly distilled water and make up to 10 ml.

VIII. Steroid sulphatase (200 units*)/ml.):
Suspend the commercial preparation with vigorous stirring (froth should be avoided) in tris-acetate buffer (solution VI). Steroid sulphatase is insoluble, therefore stir the suspension thoroughly before each assay.

*) Definition of the units, see Section B, p. 466.

Stability of the solutions

Prepare the naphthoquinone sulphonic acid solution (V) freshly for each series of measurements. Discard the benzidine solution if it becomes yellow. The enzyme suspension keeps for 3—4 weeks at 4° C. All the other solutions are stable virtually indefinitely at room temperature.

Procedure

Experimental material

See Section D I., p. 468.

Assay

The correct functioning of the assay system is first checked with 0.1 ml. steroid sulphate solution (VII) instead of the sample.

Enzymatic reaction

Pipette into two *Warburg* manometer vessels:

1. 0.20 ml. tris-acetate buffer (solution VI)
   0.10 ml. sample
   0.50 ml. steroid sulphatase suspension (VIII)
   doubly distilled water to 1.00 ml.
2. only 0.50 ml. steroid sulphatase suspension (VIII)

Pipette into a test tube:

3. all the solutions contained in 1. except for the steroid sulphatase.

Shake vessels 1 and 2 in a Warburg bath for 16 hours at 37° C. Incubate tube 3 for 16 hours in water bath at 37° C. Cool all the solutions in an ice bath and then combine the contents of 2 and 3 to give "solution 2". To solutions 1 and 2 add

1.0 ml. trichloroacetic acid solution (II)
2.0 ml. doubly distilled water,

mix and centrifuge in conical centrifuge tubes for 15 min. at 1000 to 2000 g. Without disturbing the sediment remove

2 ml. of each of the clear supernatants

for the colour reaction.

Colour reaction

Wavelength: 546 mμ; light path: 1 cm.; measure against a blank (composition: as for the standards, but containing doubly distilled water instead of the sulphate standard solution).

Standards: Pipette into centrifuge tubes with round-bottoms:

0.01—0.10 ml. sodium sulphate standard solution (I)
1.49—1.40 ml. doubly distilled water
0.5 ml. trichloroacetic acid solution (II).

Mix thoroughly and analyse as for the samples.

Samples: Pipette into centrifuge tubes with round-bottoms:

2.00 ml. deproteinized supernatant or standard solution
4.5 ml. ice-cold benzidine solution (III).

Mix thoroughly and allow to stand for exactly 1 hour in an ice bath. The level of liquid in the ice bath should be higher than the level of the liquid in the tubes. Centrifuge for 15 min.

at 0 to 4°C and 4000 g. Carefully decant the supernatant without disturbing the sediment, invert the tubes for 3 min. so that all the fluid is drained off. Return the tubes to the ice bath and add

7.0 ml. ice-cold acetone.

Shake vigorously until the sediment is evenly suspended. Centrifuge for 15 min. at 0 to 4°C and 4000 g. Carefully decant off the supernatant without disturbing the sediment. Invert the tubes and allow to stand for *ca.* 10 min. until the residue is dry. Add

0.50 ml. borate solution (IV),

warm carefully over a small flame, after complete solution of the sediment, add

5.00 ml. doubly distilled water and
0.50 ml. naphthoquinone sulphonic acid solution (V).

After exactly 10 min. add

1.0 ml. acetone.

Read the optical density at 546 mμ against the blank.

Calculations

Plot the optical densities of the standards against the μmoles sulphate/tube. Obtain the sulphate content of the samples from this standard curve. According to our standard curves 0.590 μmoles sulphate in the colour reaction gives an optical density of 1.0. Therefore instead of preparing a standard curve the results can be obtained with sufficient accuracy by assuming:

$$E \times 0.59 = \mu\text{moles sulphate taken for the colour reaction.}$$

To obtain the μmoles steroid sulphate/ml. sample the following formula is used:

$$(E_1 - E_2) \times 0.59 \times 10 \times 2 = \mu\text{moles steroid sulphate/ml. sample}$$

where

E_1 = optical density of the sample hydrolysed with sulphatase

E_2 = optical density of the sample not hydrolysed with sulphatase

10 = conversion from the 0.1 ml. sample taken for the enzymatic reaction to 1.0 ml.

2 = conversion from 2 ml. (= portion of the enzymatic reaction used for the colour reaction) to 4 ml. (= total volume of the enzymatic reaction mixture).

Example

0.1 ml. fresh, boiled urine was hydrolysed. The following values were measured:

Sample 1. (after enzymatic hydrolysis): $E_1 = 0.600$
Sample 2. (without enzymatic hydrolysis): $E_2 = 0.150$ } $E_1 - E_2 = 0.450$

$0.45 \times 0.59 \times 10 \times 2 = \mu$moles steroid sulphate/ml. urine.

Sources of Error

Sulphatase is inhibited by an excess of inorganic salts, phosphate and androsterone sulphate (10^{-2} M). Chloride and phosphate in concentrations $> 5 \times 10^{-4}$ M interfere with the colour reaction.

III. Phosphate

Principle

The phosphate liberated after the action of phosphatase is determined according to *Lohman* and *Jendrassik*[24] or *Fiske* and *Subbarow*[25] (modified according to[23]). The difference in the free phosphate before and after the action of the phosphatase gives the amount of steroid-bound phosphate, providing that no other phosphate esters capable of reacting with phosphatase are present.

Reagents

1. Sulphuric acid, A. R., conc.
2. Ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, A. R.
3. Potassium bisulphite, $K_2S_2O_5$, A. R.
4. Sodium sulphite, Na_2SO_3, A. R.
5. *p*-Methylaminophenol sulphate (Metol)
6. Sodium acetate $\cdot 3\ H_2O$, A. R.
7. Trichloroacetic acid
8. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
9. Tris-hydroxymethyl-aminomethane, tris
10. Acetic acid, glacial
11. Prednisolone monophosphate
    sodium salt.
12. Phosphatase
    Acid phosphatase*) from yeast or use the steroid sulphatase from Schering, Berlin (Germany), which is contaminated with alkaline phosphatase.

Purity of the enzyme preparation

The acid phosphatase prepared according to *) is practically free from interfering contaminants.

Preparation of Solutions

I. Ammonium molybdate solution:
Carefully pour 40.4 ml. conc. H_2SO_4 in a thin stream, with stirring, into 109 ml. doubly distilled water and allow to cool. Dissolve 12.5 g. finely powdered $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ in about 100 ml. doubly distilled water and, with stirring, pour into the solution 125 ml. of the dilute H_2SO_4. When the solution is quite cold dilute to 250 ml. with doubly distilled water.

II. Metol reducing solution:
Dissolve 25 g. finely powdered $K_2S_2O_5$ in 60 ml. doubly distilled water. Dissolve 0.2 g. *p*-methylaminophenol sulphate in 10 ml. doubly distilled water, mix the two solutions and make up to 100 ml. with distilled water. Filter through a fluted paper (discard the first 20 ml.) and store in a brown bottle.

III. Sodium acetate (2.5 M):
Dissolve 85 g. sodium acetate $\cdot 3\ H_2O$ in doubly distilled water and make up to 250 ml.

IV. Steroid phosphate standard solution (10^{-2} M):
Dissolve 48.4 mg. prednisolone monophosphate (Na salt) in doubly distilled water and make up to 10 ml.

V. Phosphate standard solution (3.22×10^{-3} M):
Dissolve 4.38 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml. Just before use dilute 10 ml. of this to 100 ml. with doubly distilled water.

*) The acid phosphatase was obtained from Dr. *Ohlenbusch*, Physiologisch-chemisches Institut der Universität Kiel, Germany.

VI. Tris-acetate buffer (1 M tris; pH 4.5):
Dissolve 242 g. tris-hydroxymethyl-aminomethane in doubly distilled water and make up to 1000 ml. Dilute 100 ml. of this solution with *ca.* 20 ml. doubly distilled water, adjust to pH 4.5 (glass electrode) by the dropwise addition of glacial acetic acid and dilute to 200 ml. with doubly distilled water.

VII. Trichloroacetic acid (20% w/v):
Dissolve 20 g. trichloroacetic acid in doubly distilled water and make up to 100 ml.

VIII. Phosphatase (600 units*)/ml.):
Dissolve 2 mg. acid phosphatase in 0.2 ml. ice-cold tris-acetate buffer (solution VI) and dilute to 10 ml. with doubly distilled water. Use the steroid sulphatase suspension described on p. 466 if an alkaline phosphatase is required (contaminant of the sulphatase preparation), and use an alkaline buffer.

Stability of the solutions

All the solutions are stable for at least 3 weeks at 4° C. The growth of bacteria or moulds in the enzyme solutions make them unusable.

Procedure

Experimental material

See Section D I., p. 468.

Assay

The correct functioning of the assay system is first checked with 0.1 ml. steroid phosphate standard solution (IV) instead of the sample.

Enzymatic reaction

Pipette into three test tubes:

1. 0.20 ml. tris-acetate buffer (solution VI)
   0.10 ml. sample
   0.50 ml. phosphatase solution (VIII)
   doubly distilled water to 1.0 ml.
2. All the solutions with the exception of the phosphatase solution (VIII).
3. Only 0.50 ml. phosphatase solution (VIII).

Mix thoroughly and incubate for 4 hours in a constant temperature bath at 37°C. Place all the tubes in an ice bath, combine the contents of 2 and 3 to give "solution 2". To solutions 1 and 2 add

1.0 ml. trichloroacetic acid solution (VII)

and remove any precipitated protein or other turbidity by centrifuging for 10 min. at 2000 g. Add

1.0 ml. of each clear supernatant

to tubes which are graduated at 10 ml., dilute to 5 ml. with doubly distilled water and proceed with the colour reaction.

*) A unit is the amount of enzyme which liberates 1 μg. prednisolone from prednisolone monophosphate in 1 hour at 37° C[17].

Colour reaction

Wavelength: 578 mμ; light path: 2 cm.; measure against a blank (composition: as for the standards, but without the phosphate standard solution).

Standards: Pipette successively into test tubes graduated at 10 ml.:

0.10—1.0 ml. phosphate standard solution (V)
0.50 ml. trichloroacetic acid solution (VII)
doubly distilled water to 5 ml.

Analyse the solutions as for the supernatants of the samples.

Samples: Add to the tube containing the supernatant from the enzymatic reaction or the standard

1 ml. ammonium molybdate solution (I)
0.5 ml. metol reducing solution (II),

mix and after exactly 10 min. add

2 ml. sodium acetate solution (III).

Dilute to 10 ml. with doubly distilled water and mix thoroughly.
After exactly 5 min. read the optical density at 578 mμ against the blank.

Calculations

Plot the optical densities of the standards against the μmoles phosphate/tube. From this standard curve obtain the phosphate content of the samples. According to our standard curves 2.1 μmoles phosphate in the colour reaction gives an optical density of 1.0. Therefore instead of preparing a standard curve the results can be obtained with sufficient accuracy by assuming:

$$E \times 2.1 = \mu\text{moles phosphate taken for the colour reaction.}$$

To obtain the μmoles steroid phosphate/ml. sample the following formula is used:

$$(E_1 - E_2) \times 2.1 \times 10 \times 2 = \mu\text{moles steroid phosphate/ml. sample.}$$

where

E_1 = optical density of the sample hydrolysed with phosphatase

E_2 = optical density of the sample not hydrolysed with phosphatase

10 = conversion from the 0.1 ml. sample taken for the enzymatic reaction to 1.0 ml.

2 = conversion from 1 ml. (= portion of the supernatant used for the colour reaction) to 2 ml. (= total volume of the supernatant).

Example

0.1 ml. fresh, boiled urine was analysed. The following values were measured:

Sample 1. (after enzymatic hydrolysis): $E_1 = 0.400$
Sample 2. (without enzymatic hydrolysis): $E_2 = 0.100$
$E_1 - E_2 = 0.300$

$0.3 \times 2.1 \times 10 \times 2 = 12.6$ μmoles steroid phosphate/ml. urine.

Sources of Error

Sulphate (10^{-1} M) and phosphate (2×10^{-2} M) inhibit the enzymatic reaction.