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**Assay of Δ^4 -3-oxosteroids and their conjugates by
the isonicotinic acid hydrazide method***

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Previous studies with rat liver microsomal and soluble fractions demonstrated the presence of enzyme systems capable of reducing the Δ^4 double bond of testosterone 17-glucosiduronate, testosterone 17-N-acetylglucosaminide, and testosterone 17-sulfate to the corresponding 5α - and 5β -reduced steroid conjugates. Following incubation of the radioactive conjugates with liver enzymes, the metabolites were separated and purified by a combination of column chromatography, hydrolysis of the conjugates, and identification of the liberated aglycones. For continuation of the enzymic studies, a simple and sensitive method for the analysis of Δ^4 -3-oxosteroids and their conjugates is needed. The spectrophotometric methods for the assay of the Δ^4 -hydrogenase activity are based on the measurement of the absorption either of the Δ^4 -3-oxo grouping or of the hydrazone formed by condensation of the Δ^4 -3-oxosteroid with isonicotinic acid hydrazide (INH), after the steroid is extracted with organic solvents such as methylene chloride. However, these methods are not applicable to steroid conjugates, because the conjugates are not extracted with such solvents. This paper describes a specific, sensitive, and simple method for the quantitative estimation of Δ^4 -3-oxosteroids and their conjugates using the INH method.

Procedures. Standard curves were prepared in the following manner: To 2.0 ml of the assay medium, which consisted of 3.0 ml of 0.25 M sucrose solution and 3.5 ml of 0.2 M Tris-maleate buffer (pH 6.5), was added methanol solution of the steroid or steroid conjugate (15–260 nmol) and made up to 10 ml with methanol to afford the buffer-methanol solution. Then, 3.0 ml of this solution was evaporated to dryness *in vacuo*. The residue was dissolved in 3.0 ml of the INH reagent [200 mg of INH is dissolved in 100 ml of acetic acid-methanol reagent (4:1)] and kept at 37°C for 2 hr. The absorbance at 372 nm was determined with a reference consisted of the test reagent with the solvent blank. Recoveries of the steroids from the incubation medium were carried out as follows: 3.0 ml of the microsomal or soluble fraction was added to 3.5 ml of 0.2 M Tris-maleate buffer (pH 6.5), containing an NADPH-generating system. To this enzyme medium, which was kept at 4°C in an ice bath, was added a methanol solution of the steroid or a 50% methanol solution of the steroid conjugate (0.2–3.0 μ mol, 100 μ l), followed by thorough mixing. Two milliliters of this incubation medium was removed and immediately made up to 10 ml with methanol. The resultant

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mixture was allowed to stand at room temperature at least for 1 hr and centrifuged at 3000 rpm for 10 min. Three milliliters of the supernatant was evaporated to dryness *in vacuo*. The residue was treated with the INH reagent, and the absorbance was determined as described above.

The absorption spectrum of testosterone 3-isonicotinic acid hydrazone formed in the standard analysis revealed an absorption maximum at 372 nm. The maximal molar extinction coefficient of testosterone after color development was 14800 and was in good accord with the value of 15000 obtained for the synthesized testosterone 3-isonicotinic acid hydrazone, whose spectrum was taken in the standard condensation medium. Therefore, it became apparent that testosterone reacted almost quantitatively with INH. The color development was little affected by the assay buffer system such as 0.2 M Tris-maleate buffer and 0.2 M Tris-HCl buffer, which have been used as the assay medium for Δ^4 -hydrogenases.

In the standard analysis, Δ^4 -3-oxosteroids such as testosterone, androstenedione, progesterone, corticosterone, and hydrocortisone gave molar extinction coefficients of about 15000, these values being about 25% higher than those reported by Umberger. Introduction of the 2- or 6-hydroxy (or oxo) grouping into Δ^4 -3-oxosteroids resulted in somewhat lower color intensities. The high specificity for Δ^4 -3-oxosteroids was demonstrated by little or no color development of ring A saturated 3- or 17-oxosteroids and 3,17-dihydroxysteroids.

Good recoveries of testosterone and testosterone conjugates added to the rat liver microsomal and soluble fractions proved the validity of this method.

The catabolic sequence of Δ^4 -3-oxosteroids is well established and proceeds mainly via 4,5-dihydro-3-oxosteroids to saturated 3-hydroxysteroids by the consecutive action of Δ^4 -hydrogenases and 3-oxosteroid-oxidoreductases. Thus, this method is especially applicable to the kinetic studies of these enzyme systems, employing Δ^4 -3-oxosteroids and their conjugates as substrates.