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The determination of hydroxysteroids as their 3,5-dinitrobenzoates*

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[Received for publication February 26, 1960]

► Interest in the physiological and pathological significance of hydroxylated steroids has led to the development of several methods for their determination, some of which have been reviewed by Engel and Baggett (1) and by Weinmann and Jayle (2). The

* Equipment used in this work was purchased from a Rockefeller Foundation grant.

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method proposed by Kellie *et al.* (3) is based on the esterification of steroid alcohols with 3,5-dinitrobenzoic acid and the subsequent measurement of the color produced when the esters are treated with dilute alkali in the presence of acetone. This is probably the best method concerning specificity, quantitative recovery, and reproducibility, and its originators further indicated that with microcells it could be used to estimate very small amounts of esters. They also showed that it could be combined with chromatographic separation of the 3,5-dinitrobenzoates to provide useful data on the composition of the nonketonic alcohol fraction of steroids extracted from normal and abnormal urine (4).

In the course of some studies on *in vitro* steroid hydroxylation, it soon became apparent to us that the sensitivity of the method had to be increased. The choice of volume for diluting the reaction mixture after color development is not arbitrary, as was assumed by Kellie *et al.* (3), and the concentration and solvent used for the alkali reagent appear to be critical. Consequently, we determined the conditions to carry out a stable color reaction in a final volume of 1 ml.

The experiments described as follows were conducted on esters prepared as described originally (3), with the exception that esterification was completed with 5 minutes of heating, instead of 30 seconds. The 3,5-dinitrobenzoyl chloride was obtained according to Vogel (5). This reagent was kept in a vacuum dessicator over P_2O_5, and frequent determinations of its melting point, 67.5°-69.0°, showed no indication of decomposition. Some commercial samples of this reagent gave erratic results because of hydrolysis in various degrees. Three steroids from commercial sources, cholesterol, pregnanediol, and estriol, were used as received. A Beckman D.U. spectrophotometer was used throughout for the colorimetric determinations.

In the original procedure (3), a portion of the steroid ester equivalent to 0.08 mg of 3,5-dinitrobenzoyl radical is dissolved in 0.8 ml of acetone, and 0.2 ml of 0.1% KOH in ethanol is added. After 5 minutes at room temperature, 9 ml of acetone or ethanol is added and the diluted sample is read in the region between 550 to 570 mμ. Turbidity and rapid fading of the color occurred, however, in the more concentrated mixtures when lesser volumes of acetone were used in the final dilution. Although the initial readings obtained with alkali in different mixtures of water and ethanol or acetone were approximately the same, the figures shown in Table 1 indicated that aqueous alkali prevented turbidity and gave a more stable color than that developed by the other mixtures. Some experiments suggested that there might be an optimum concentration of KOH, as far as stability of the color was concerned. Accordingly, several concentrations of aqueous KOH were tested and the results are shown in Table 2. It can be seen that 0.02% KOH in water develops a quite stable color.

The final procedure adopted for the colorimetric determination of the steroid esters is as follows: An

TABLE 1. STABILITY OF COLOR WITH 0.1 PER CENT KOH IN DIFFERENT SOLVENTS *

Solvents †	Minutes After Mixing		T ‡
	5	55	
Water	0.469	0.375	−
25% ethanol	0.460	0.310	−
50% ethanol	0.472	0.300	+
75% ethanol	0.472	0.298	+
Ethanol	0.471	0.355	+
25% acetone	0.469	0.281	−
50% acetone	0.451	0.229	+
75% acetone	0.431	0.200	+

* Figures are absorbance values at 555 mμ in 1 cm cells. The reaction mixture consists of 0.8 ml of an acetone solution of cholesteryl 3,5-dinitrobenzoate (1 mg/100 ml of 3,5-dinitrobenzoyl radical) and 0.2 ml of 0.1% KOH in the appropriate solvent.

† Volume per cent of solvent in aqueous solution.

‡ Presence of turbidity in the first half hour.

TABLE. 2. STABILITY OF COLOR WITH DIFFERENT CONCENTRATIONS OF AQUEOUS KOH *

Concentrations	Minutes After Mixing		
	5	125	365
%			
0.100	0.487	0.305	0.220
0.075	0.512	0.374	0.215
0.050	0.490	0.429	0.336
0.035	0.490	0.471	0.440
0.030	0.486	0.454	—
0.025	0.489	0.471	—
0.020	0.481	0.493	—

* See Table 1 for composition of final mixture. Only the concentration of aqueous KOH was varied.

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aliquot of 0.8 ml of an acetone solution of the steroid ester containing about 8 μg of 3,5-dinitrobenzoyl radical was treated with 0.2 ml of 0.02% aqueous KOH. After 5 minutes at room temperature, the color was read at maximum absorbancy of 555 mμ. This procedure compared favorably with the original method in simultaneous determinations carried out on the 3,5-dinitrobenzoyl esters of cholesterol, pregnanediol, and estriol. Since the final volume is one-tenth that of the original procedure, the sensitivity is correspondingly increased. Table 3 shows this comparison. The color

TABLE 3. ABSORBANCE/μMOLE * OF ESTERIFIED STEROIDS

Steroid	A †	B ‡	C §
Cholesterol	0.97	0.94	1.08
Pregnanediol	1.86	1.84	2.27
Estriol	2.83	2.69	not given
Average/μmole/ No. of OH groups	0.94	0.92	1.12 ‖

* This is calculated by the following formula:

$$E \times d \times \frac{M.W.}{S} \times 10^{-6}$$

in which E = absorbance (1 cm path) at maximum, d = dilution factor, $M.W.$ = molecular weight, and S = mg of steroid used in the preliminary esterification (3).

† Our data by original procedure (3).

‡ Our data by modified procedure.

§ Original data (3).

‖ Calculated from original data.

obeyed Beer's law, at least in the range of 0.8 to 8 μg of 3,5-dinitrobenzoyl radical, the average absorbance for 1 μg (1 cm path of light) being 0.062. The modified reaction is useful for the analysis of effluents in column chromatography, or for eluates from paper chromatograms of the esters.

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