[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, COLLEGE OF MEDICINE, UNIVERSITY OF UTAH]

The Reaction of Adrenocortical Hormones With 2,4-Dinitrophenylhydrazine[1]

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The bisdinitrophenylhydrazones of desoxycorticosterone, Δ^4-pregnene-17α,21-diol-3,20-dione, cortisone, and cortisol have been prepared and characterized. The 21-monoacetates of these four adrenocortical steroids have been converted to their 3-monodinitrophenylhydrazones which are suitable derivatives for spectrophotometric estimation.

Due to their intense colors and their characteristic ultraviolet absorption spectra the dinitrophenylhydrazones are generally regarded as the most suitable derivatives of steroid ketones. It was the aim of the present investigation to find out if they can also be used for the characterization of adrenocortical hormones.

Since 21-hydroxypregnenolone is known to react readily with dinitrophenylhydrazine in alcoholic hydrochloric acid,[3] the same was to be expected for desoxycorticosterone. When the reaction was carried out at room temperature, two moles of the reagent were consumed and the bisdinitrophenylhydrazone was obtained in high yield. No noticeable amounts of osazone were formed under these conditions. An osazone was formed, however, when the reaction mixture was refluxed, although the formation proceeded rather slowly in comparison with 21-hydroxypregnenolone. Thus after two hours the dinitrophenylhydrazine utilized amounted to only 2.7 moles corresponding to 35% osazone. The reason for this difference is probably the relative insolubility of the intermediate bisdinitrophenylhydrazone in alcohol. On the contrary, the dinitrophenylhydrazone of 21-hydroxypregnenolone is quite soluble in alcohol, so it can react further with dinitrophenylhydrazine.

It has been shown recently[3] that Δ^5-pregnene-3β,17α,21-triol-20-one gives only a low yield of the normal dinitrophenylhydrazone and that the main reaction product is the dinitrophenylhydrazone of $\Delta^{5,16}$-pregnadiene-3β,21-diol-20-one which is formed by dehydration. When Δ^4-pregnene-17α,21-diol-3,-20-dione, cortisone, and cortisol (Kendall's compound F) were allowed to react with dinitrophenylhydrazine in alcoholic hydrochloric acid, the dinitrophenylhydrazones of the former two precipitated immediately, that of cortisol only after prolonged standing. Since all three dinitrophenylhydrazones showed an absorption maximum between 380 and 382 mμ, it can be assumed that they still contained the 17α-hydroxyl group, *i.e.* that no dehydration had taken place. This also followed from the analysis of cortisol bisdinitrophenylhydrazone. The dehydration products, the bisdinitrophenylhydrazones of the 4,16-doubly unsaturated compounds, should exhibit a maximum at 388–389 mμ. That they were not formed, or only in minute quantities, is probably due to the insolubility of the normal bisdinitrophenylhydrazones in alcohol. The 21-monoacetates of the three dinitrophenylhydrazones mentioned above showed a maximum at 376 mμ after purification by chromatography. Each acetate was accompanied by a very small amount of a by-product which had a maximum at 382 mμ and possibly represented the acetate of the doubly unsaturated bisdinitrophenylhydrazone.

It has previously been found that the 20-keto group of 21-acetoxypregnenolone does not react with dinitrophenylhydrazine, when the reaction is carried out in chloroform-acetic acid.[3] Consequently desoxycorticosterone acetate, under the same conditions, gave only the 3-monodinitrophenylhydrazone.[4] In a similar manner, the 3-monodinitrophenylhydrazones of Δ^4-pregnene-17α,21-diol-3,20-dione 21-monoacetate, cortisone 21-monoacetate, and cortisol 21-monoacetate were obtained in quantitative yields. These derivatives are quite soluble in chloroform and can be estimated spectrophotometrically after excess dinitrophenylhydrazine has been removed with pyruvic acid.[4] The first two mentioned above as well as desoxycorticosterone acetate 3-monodinitrophenylhydrazone[4] show a maximum at 389–390 mμ, cortisone 21-monoacetate 3-monodinitrophenylhydrazone at 387 mμ.[5] This slight shift to shorter wave-lengths is probably caused by the 11-keto group.[6] As long as the dinitrophenylhydrazine present in the reaction mixture and later removed as pyruvic acid dinitrophenylhydrazone does not exceed the fifty-fold theoretical amount, the ultraviolet spectra show a sharp peak. Larger excesses cause a background absorption which lets the peak appear rather broad and shifts it to somewhat shorter wave-lengths.

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(3) Reich and Samuels, *J. Org. Chem.*, **19**, 1041 (1954).

(4) Reich, Crane, and Sanfilippo, *J. Org. Chem.*, **18**, 822 (1953).

(5) Mattox and Kendall, *J. Biol. Chem.*, **188**, 287 (1951).

(6) Dorfman, *Chem. Revs.*, **53**, 47 (1953).

EXPERIMENTAL[7]

Desoxycorticosterone bisdinitrophenylhydrazone. (*a*) A mixture of 12.8 mg. of desoxycorticosterone and 24.1 mg. of dinitrophenylhydrazine (3.1 moles) in 8 cc. of abs. ethanol and 6 drops of conc'd hydrochloric acid was kept at room temperature overnight. Then 200 mg. of potassium acetate and 3 drops of acetone were added. The mixture was extracted with chloroform after 3½ hours and worked up in the usual manner.[4] The acetone dinitrophenylhydrazone purified by two successive chromatographies amounted to 10.1 mg. which corresponds to a consumption of 2.04 moles of dinitrophenylhydrazine.

(*b*) A mixture of 42.8 mg. of desoxycorticosterone and 60 mg. of dinitrophenylhydrazine in 7.2 cc. of abs. ethanol and 14 drops of conc'd hydrochloric acid was allowed to stand for 2½ hours. The precipitate was filtered, washed with ethanol, and recrystallized from chloroform-ethanol. The red crystals melted at 251–254°; maxima at 257 mμ and 383 mμ (ε 29,838 and 53,322), minimum at 313 mμ (ε 6,769).

Anal. Calc'd for $C_{33}H_{38}N_8O_9$: N, 16.23. Found: N, 16.52.

Acetate. A sample of the preceding bisdinitrophenylhydrazone was dissolved in a mixture of pyridine and acetic anhydride 2:1 and allowed to stand overnight. After addition of ice, the precipitate was filtered, washed with water, dried, and chromatographed. Most of the material was eluted with benzene. The orange crystals which were obtained from chloroform-ethanol melted at 234–236°; maxima at 256 mμ and 378 mμ (ε 32,312 and 49,740), minimum at 310 mμ (ε 8,778); infrared band at 5.68 mμ.

Anal. Calc'd for $C_{35}H_{40}N_8O_{10}$: N, 15.30. Found: N, 15.06.

Δ⁴-Pregnene-3,20-dione-21-al trisdinitrophenylhydrazone. A mixture of 25.2 mg. of dinitrophenylhydrazine and 10.2 mg. of desoxycorticosterone in 7 cc. of abs. ethanol and 6 drops of conc'd hydrochloric acid was refluxed for 2 hours. The crystals which precipitated on cooling were washed with ethanol, chromatographed (eluted with benzene-chloroform 4:1), and recrystallized from chloroform-ethanol; m.p. *ca.* 215°; maximum at 394 mμ (Cary spectrophotometer). The substance gave a purple color reaction with alcoholic NaOH.

Anal. Calc'd for $C_{39}H_{40}N_{12}O_{12}$: N, 19.35. Found: N, 18.87.

Δ⁴-Pregnene-17α,21-diol-3,20-dione 21-monoacetate bisdinitrophenylhydrazone. A solution of 25 mg. of dinitrophenylhydrazine in 3 cc. of abs. ethanol and 6 drops of conc'd hydrochloric acid was added to 10 mg. of "substance S," dissolved in ethanol. Red crystals appeared almost immediately. After standing overnight, the excess reagent was destroyed with Benedict's reagent.[4] The crude dinitrophenylhydrazone which showed a maximum at 381 mμ was acetylated with 1 cc. of pyridine and 0.5 cc. of acetic anhydride. After standing at room temperature overnight, ice was added and the precipitate was filtered, washed, dried, and chromatographed. Benzene-chloroform 9:1 eluted a yellow band which had a maximum at 382 mμ. The fractions eluted with benzene-chloroform 7:3 were recrystallized from chloroform-ethanol and gave red-orange crystals of m.p. 200–203°; maxima at 257 mμ and 376 mμ (ε 29,616 and 46,275), minimum at 310 mμ (ε 8,908).

Anal. Calc'd for $C_{35}H_{40}N_8O_{11}$: C, 56.14; H, 5.39. Found: C, 56.35; H, 5.67.

Δ⁴-Pregnene-17α,21-diol-3,20-dione 21-monoacetate 3-monodinitrophenylhydrazone. A mixture of 21.0 mg. of Δ⁴-pregnene-17α,21-diol-3,20-dione 21-monoacetate and 20.2 mg. of dinitrophenylhydrazine was suspended in 12 cc. of chloroform and 2 cc. of glacial acetic acid. After standing overnight and addition of 3 drops of acetone, the solution was kept for 2½ hours, diluted with chloroform, washed with sodium carbonate solution and water, dried, and evaporated. The residue was chromatographed on 6 g. of aluminum oxide. The acetone dinitrophenylhydrazone which was eluted first weighed 12.0 mg. corresponding to 10.0 mg. of dinitrophenylhydrazine. Thus 10.2 mg. of the latter had been utilized, *i.e.* 0.95 mole. The fractions eluted with benzene-chloroform 7:3 to 1:1 were recrystallized from chloroform-ethanol. The red dinitrophenylhydrazone melted at 231–234°; maximum at 389 mμ.

Anal. Calc'd for $C_{29}H_{36}N_4O_8$: N, 9.86. Found: N, 9.99.

Cortisol bisdinitrophenylhydrazone. This derivative was made from 10 mg. of cortisol and 25 mg. of dinitrophenylhydrazine in 3.5 cc. of abs. ethanol containing 6 drops of conc'd hydrochloric acid. After standing overnight, the crystals were filtered and washed with cold ethanol. They decomposed at 270°; maximum at 382 mμ.

Anal. Calc'd for $C_{33}H_{38}N_8O_{11}$: N, 15.51. Found: N, 15.37.

21-Monoacetate. Cortisol bisdinitrophenylhydrazone (12 mg.) was dissolved in 1 cc. of pyridine and 0.5 cc. of acetic anhydride and allowed to stand overnight. After addition of ice, the precipitate was filtered, washed with water, dried, and chromatographed. The fractions eluted with benzene-chloroform 9:1 showed a maximum at 382 mμ; those eluted with benzene-chloroform 7:3 were recrystallized from dilute ethanol and melted at 236–240°; maxima at 259 mμ and 376 mμ (ε 27,175 and 42,715), minimum at 310 mμ (ε 8,574).

Cortisol 21-monoacetate 3-monodinitrophenylhydrazone. This dinitrophenylhydrazone was prepared from 18.0 mg. of cortisol 21-monoacetate and 25.5 mg. of dinitrophenylhydrazine in 15 cc. of chloroform and 2.5 cc. of glacial acetic acid. The next day the mixture was worked up as described for Δ⁴-pregnene-17α,21-diol-3,20-dione 21-monoacetate 3-monodinitrophenylhydrazone. The reagent utilized was calculated from the weight of the acetone dinitrophenylhydrazone isolated by chromatography (19.8 mg.) and amounted to 1.0 mole. The second substance, eluted with benzene-chloroform 2:3 and 1:4, was recrystallized from chloroform-ethanol. The red crystals thus obtained softened at 218° and melted at 235–240°; maximum at 389 mμ.

Anal. Calc'd for $C_{29}H_{36}N_4O_9$: N, 9.59. Found: N, 9.96.

Cortisone bisdinitrophenylhydrazone.[8] A solution of 25.0 mg. of dinitrophenylhydrazine in 3 cc. of abs. ethanol and 6 drops of conc'd hydrochloric acid was added to a solution of 10.0 mg. of cortisone in 1 cc. of abs. ethanol. A precipitate appeared immediately. The next day it was filtered, washed with ethanol, and dried. It weighed 19.0 mg. and decomposed between 275 and 280°; maximum at 380 mμ. This dinitrophenylhydrazone could not be recrystallized satisfactorily and obtained analytically pure.

Potassium acetate (100 mg.) and two drops of acetone were added to the mother liquor. After one hour the mixture was diluted with chloroform, washed with sodium carbonate solution and water, dried, and evaporated. The residue was chromatographed on 3 gm. of aluminum oxide. The acetone dinitrophenylhydrazone thus isolated weighed 17.1 mg. The dinitrophenylhydrazine utilized amounted to 1.95 moles.

21-Monoacetate. Cortisone bisdinitrophenylhydrazone (11 mg.) was acetylated with 1 cc. of pyridine and 0.5 cc. of acetic anhydride. After standing overnight and addition of ice, the precipitate was filtered, washed with water, dried, and chromatographed. The fractions eluted with benzene-chloroform 7:3 to 1:1 were recrystallized from chloroform-

(7) All melting points were taken on a Kofler micro hot stage and are corrected. The ultraviolet spectra were taken in chloroform in a Beckman spectrophotometer Model DU, the infrared spectrum in chloroform in a Perkin-Elmer spectrophotometer Model 21. The microanalyses were carried out by Drs. Weiler and Strauss, Oxford, England, and by Huffman Microanalytical Laboratories, Wheatridge, Colorado. The aluminum oxide used in all experiments was acid-washed and was kindly supplied by Dr. H. B. McPhillamy, Ciba Pharmaceutical Products, Summit, New Jersey.

(8) Cortisone 3-monodinitrophenylhydrazone was prepared by Mason, Myers, and Kendall, *J. Biol. Chem.*, 114, 613 (1936).

ethanol and melted at 201–203°; maxima at 258 mμ and 376 mμ (ε 27,801 and 43,227), minimum at 310 mμ (ε 9,408).

Quantitative determination of cortisone acetate. Alcoholic solutions containing 1.0, 0.5, 0.25, 0.125, and 0.0625 mg. of cortisone acetate were placed in five small test tubes and evaporated. One cc. of a solution of 20 mg. of dinitrophenylhydrazine in 12 cc. of chloroform and 2 cc. of glacial acetic acid (dissolved by refluxing for 8 minutes) was added to each tube. The mixtures were allowed to stand overnight and again for one hour after addition of a small drop of pyruvic acid. They were diluted with chloroform, washed with sodium carbonate solution and water, concentrated, and filled up to 10 cc. Amounts of 0.19, 0.375, 0.75, and 1.5 cc. were withdrawn from the first to fourth tubes and were diluted with chloroform to 3 cc. The spectra of these solutions were measured as well as that of 3 cc. of solution originating from the fifth tube. The results are summarized in Table I.

TABLE I

DETERMINATION OF CORTISONE ACETATE

Tube	Cortisone acetate, mg.	DNP* applied, moles	Maximum of dinitrophenylhydrazone, mμ	Optical densities (relative to 1)
1	1.0	2.9	387	(1.000)
2	0.5	5.8	387	1.002
3	0.25	11.6	387	1.041
4	0.125	23.2	386	1.0045
5	0.0625	46.4	385	1.143
	0		360**	

* DNP = dinitrophenylhydrazine.
** Background absorption.

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