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THE REACTION OF STEROID KETONES WITH 2,4-DINITROPHENYLHYDRAZINE[1, 2]

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The present work deals with the formation and quantitative isolation of steroidal dinitrophenylhydrazones and includes a study of their chromatographic behavior and absorption characteristics. The specific properties of these derivatives should be useful in the identification of ketosteroids isolated from natural sources.

The reaction leading to the formation of dinitrophenylhydrazones is usually carried out in alcoholic solution in the presence of small amounts of a mineral acid. Under the conditions specified in the experimental part steroids containing keto groups in the 3-, 6-, 7-, 12-, 16-, 17-, and 20-positions reacted rapidly with dinitrophenylhydrazine. The 11-keto group is known to be unreactive to all carbonyl reagents. Side reactions such as dehydrations were not observed, but keto acids of the bile acid type were esterified, and the dinitrophenylhydrazones of the corresponding esters were obtained (1).

The dinitrophenylhydrazones of most ketosteroids are so insoluble in ethanol that they precipitate from the reaction mixture and can be separated by filtration. In the case of progesterone, for instance, the amount of bisdinitrophenylhydrazone thus obtained is almost quantitative (2). However, complete isolation of dinitrophenylhydrazones which are partly soluble in alcohol is difficult, since addition of water to the reaction mixture causes precipitation of both dinitrophenylhydrazone and dinitrophenylhydrazine. The latter must be used in excess especially when the quantities of ketosteroid to be estimated are less than one mg. In our hands removal of excess reagent by chromatography as previously suggested (3, 4) proved to be time-consuming and incomplete. Oxidation with Benedict's reagent, however, a method used for the quantitative determination of Δ^5-pregnene-3β-ol-20-one and progesterone (5), was found to be simple and widely applicable. It has been used successfully in the present work for the isolation of various dinitrophenylhydrazones (Method A 1).

In order to determine the amount of dinitrophenylhydrazine utilized in the reaction, two methods have been developed. One (Method A 2) involves conversion of the excess dinitrophenylhydrazine to acetone dinitrophenylhydrazone, the other (Method A 3) to pyruvic acid dinitrophenylhydrazone. The former can easily be separated by chromatography and the latter by extraction with sodium

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carbonate solution. The weights of these derivatives can be estimated gravimetrically or spectrophotometrically and represent the amount of dinitrophenylhydrazine not utilized in the reaction (Table I). For an unknown ketosteroid the number of reactive carbonyl groups can thus be determined. Application of either method is also indicated, if the ketosteroid contains a carboxylic acid ester group which is sensitive to the alkaline Benedict's reagent.

TABLE I

THE REACTION OF DINITROPHENYLHYDRAZINE WITH VARIOUS KETOSTEROIDS ESTIMATED BY ISOLATION OF THE EXCESS REAGENT

COMPOUND	METHOD (see text)	WEIGHT OF STEROID, mg.	DNPH[a] ADDED, mg.	ACETONE DNP,[b] mg.	PYRUVIC ACID DNP,[b] mg.	EXCESS DNPH,[a,c] mg.	DNPH[a] REACTED WITH STEROID		
							Found, mg.	Calc'd as mono DNP,[b] mg.	Yield, %
Δ^5-Androstene-3β,17β-diol-16-one	A 2	10.0	19.5	16.0	—	13.3	6.2	6.52	95.2
Allopregnane-3β,17α-diol-20-one 3-acetate	B 1	15.0	16.26	10.1	—	8.40	7.86	7.90	99.5
Methyl Δ^9-3α-hydroxy-12-ketocholenate	A 2	15.0	16.67	12.9	—	10.72	5.95	7.40	80.0[d]
Desoxycorticosterone acetate	B 1	10.0	15.0	10.2[e]	—	9.80	5.20	5.32	98.0
3-Ketocholanic acid	B 1	15.0	15.0	8.5[f]	—	7.07	7.93	7.95	99.8
	B 1	30.0	30.0	17.0[f]	—	14.1	15.90	15.9	100.0
Methyl 3-ketocholanate	A 3	15.0	16.67	—	12.3	9.09	7.58	7.65	99.1
Δ^4-Cholestene-3-one	B 2	10.0	10.0	—	6.8	5.02	4.98	5.16	96.5
	B 2	7.14	7.14	—	4.4	3.25	3.89	3.68	105.5
Cortisone acetate	B 2	15.0	10.0	—	4.1	3.03	6.97	7.4	94.2
Δ^4-Pregnene-11β,17α,20β,21-tetrol-3-one 20,21-diacetate	B 2	15.0	15.0	—	10.9	8.05	6.95	6.62	104.8
Dehydroepiandrosterone acetate	B 2	20.0	20.0	—	12.4	9.17	10.83	12.0	90.2[d]
Pregnane-3α,12α-diol-20-one diacetate	B 2	15.0	10.0	—	6.4	4.73	5.27	7.12	74.0[d]

[a] DNPH = dinitrophenylhydrazine. [b] DNP = dinitrophenylhydrazone. [c] Calc'd from acetone or pyruvic acid DNP. [d] The reaction was probably not complete. [e] There was also isolated 1.6 mg. of dinitrophenylhydrazine monoacetate which corresponds to 1.32 mg. of DNPH. [f] After purification by chromatography.

Mattox and Kendall (6) have shown recently that certain ketosteroids react with dinitrophenylhydrazine in a mixture of chloroform and glacial acetic acid. Using these two solvents in the proportion 6:1 we found that the keto group in Δ^4-cholestene-3-one reacts more slowly than in a mixture of ethanol and conc'd hydrochloric acid. The reaction is complete, however, after standing at room temperature overnight. The same period of time does not suffice for complete reaction of the 17-keto group of Δ^4-androstene-3,17-dione or the 20-keto group

of progesterone. In these cases prolonged standing at room temperature or heating is required. The keto group in 7-ketocholesteryl acetate which is known to be sterically hindered reacts even slower with dinitrophenylhydrazine than the keto groups mentioned above. If a 20-ketosteroid contains an acetoxy group in the 21-position, no dinitrophenylhydrazone is formed in chloroform-acetic acid solution at room temperature (*cf.* 6). Thus desoxycorticosterone acetate and cortisone acetate yield only the corresponding 3-monodinitrophenylhydrazones. These findings have led to the development of a method for the quantitative estimation of adrenocortical steroids which will be published later.

When the excess of dinitrophenylhydrazine was determined as acetone dinitrophenylhydrazone (Method B 1), it was frequently observed that the alkaline washes were brown and contained some dinitrophenylhydrazine monoacetate which could be isolated after acidification. The formation of this derivative showing an absorption maximum at 325 mμ (in chloroform) is probably due to impurities in the chloroform which catalyze the acetylation of dinitrophenylhydrazine. In case that a significant quantity of this compound is apparent, the weight should be considered in calculating the amount of excess dinitrophenylhydrazine. If pyruvic acid is used for removal of this excess (Method B 2), both the pyruvic acid dinitrophenylhydrazone and the dinitrophenylhydrazine monoacetate appear in the carbonate washes and are determined together. Since their molecular weights are not appreciably different, the error in estimating the excess dinitrophenylhydrazine is insignificant.

The dinitrophenylhydrazones of keto acids can also be prepared in chloroform-acetic acid solution, thus avoiding the esterification which occurs in ethanol-hydrochloric acid solution. After addition of acetone to the reaction mixture, the keto acid dinitrophenylhydrazone and the acetone dinitrophenylhydrazone can easily be separated by extraction with sodium carbonate solution. The use of acetone is advantageous, since acetone dinitrophenylhydrazone is far more soluble in chloroform than dinitrophenylhydrazine itself.

The results of a few typical experiments are listed in Table I.

As an alternate method for removal of excess dinitrophenylhydrazine the reaction mixture was acetylated with acetic anhydride in pyridine. It was found that under these conditions the dinitrophenylhydrazine was converted to its hitherto unknown triacetate, a white compound (maximum at 300 mμ in chloroform) which is only slightly soluble in benzene, chloroform, and ethanol. Although in large-scale preparations the dinitrophenylhydrazone can be separated from the triacetate by extraction with one of these solvents, a quantitative separation of small amounts could not be accomplished, not even by chromatography. A similar method was used recently by Mattox (7). After acetylation in glacial acetic acid the dinitrophenylhydrazine monoacetate was obtained and separated due to its insolubility in chloroform.

The characteristics of several dinitrophenylhydrazones of steroid ketones are listed in Table II. All were purified by chromatography on aluminum oxide Merck except those of ketols and ketol acetates for which acid-washed aluminum oxide was employed. The solvents or solvent mixtures used for elution are noted

in column 3. As can be seen from the table, mono- and bis-dinitrophenylhydrazones of steroid ketones which do not contain a hydroxyl group are usually eluted with mixtures of hexane and benzene or pure benzene, and those of hydroxy compounds with mixtures of benzene and chloroform or pure chloroform. If the hydroxyl group can be acetylated, the resulting acetate is eluted from the column with a less polar solvent than is required for the dinitrophenylhydrazone of the free hydroxyl compound. For an unknown ketosteroid certain conclusions can be drawn from the chromatographic behavior of its dinitrophenylhydrazone. If the latter is eluted, for instance, with benzene-chloroform 4:1 before and after acetylation, it can be assumed that it contains a tertiary hydroxyl group or a hydroxyl group in the 11-position.

The melting points in Table II refer to derivatives which were recrystallized from chloroform-ethanol or pure ethanol. In many cases the amount of starting material available was so small (less than 1 mg.) that the dinitrophenylhydrazone could not be purified completely by recrystallization. For the same reasons only the absorption maxima are listed in column 5, since the accurate determination of molecular extinction coefficients was impracticable.

The dinitrophenylhydrazones of Δ^4-3-ketosteroids are known to exhibit an absorption maximum at approximately 390 mμ in chloroform (8–10). The same value was found by us for several dinitrophenylhydrazones of this type. The dinitrophenylhydrazones of two Δ^5-7-keto-, one Δ^9-12-keto-, and two Δ^{16}-20-keto-steroids, however, showed a maximum at 385 mμ. Maxima ranging from 381 mμ to 384 mμ have been reported for the dinitrophenylhydrazones of Δ^1-3-ketosteroids (10, 11, 6) and a maximum of 376 mμ for Δ^4-cholestene-6-one dinitrophenylhydrazone (12). The latter compares well with the maximum given by the dinitrophenylhydrazone of Δ^4-cholestene-3β-ol-6-one acetate. Since both maxima differ considerably from those of other α,β-unsaturated dinitrophenylhydrazones, it is possible that the dinitrophenylhydrazones of Δ^4-6-ketosteroids do not possess the normal structure, but instead may exist as pyrazoline derivatives. The absorption maximum at 390 mμ seems to be typical for the dinitrophenylhydrazones of Δ^4-3-ketosteroids. It was also noted that only the latter are bright red in color. The dinitrophenylhydrazones of the other α,β-unsaturated ketones mentioned above are orange or even yellow.

Saturated 3- and 17-ketosteroids yield dinitrophenylhydrazones with an absorption maximum at 367–369 mμ. We found the same to be true for dinitrophenylhydrazones of 20-ketosteroids which have no hydroxyl group in the 17- or 21-positions.

The bisdinitrophenylhydrazones of steroids with one saturated and one α,β-unsaturated ketone group have single absorption maxima at wave lengths between those of the maxima given by the respective monodinitrophenylhydrazones. Thus progesterone bisdinitrophenylhydrazone and Δ^4-androstene-3,17-dione bisdinitrophenylhydrazone display maxima at 380 mμ.

The presence of a 17-hydroxyl group in 20-ketosteroid dinitrophenylhydrazones causes a shift of the absorption maximum to shorter wave-lengths as exemplified by comparison of the dinitrophenylhydrazones of allopregnane-

TABLE II

CHARACTERISTICS OF DINITROPHENYLHYDRAZONES OF STEROID KETONES

DINITROPHENYLHYDRAZONE OF	METHOD (see text)	ELUTED WITH	M.P., °C.	MAX. IN $CHCl_3$ mμ
3-Ketocholanic acid	B 1	—	230–233	368
Methyl 3-ketocholanate	A 3	Hex.-benz.2:3	145–150	368
Ethyl 3-ketocholanate	(B 1)	Hex.-benz.2:3	140–147	369
Testosterone	A 1	Benz.-chlf.9:1	203[a]	390
Testosterone acetate	(A 1)	Hex.-benz.1:4	220–224	390
Methyl Δ^4-3,11-diketo-17α-hydroxyetiocholenate	A 2	Benz.-chlf.7:3	235–239	389
Methyl Δ^4-3-keto-11β,17α-dihydroxyetiocholenate	A 2	Benz.-chlf.7:3	233–247	390
Progesterone (3-mono DNP[b])	A 1	Hex.-benz.1:4	215–218/ 235–238	390
Desoxycorticosterone acetate (3-mono DNP[b])	B 1	Benz.-chlf.4:1[c]	180/ 202–205	389
Δ^4-Pregnene-11β,17α,20β,21-tetrol-3-one 20, 21-diacetate	B 2	Benz.-chlf.3:2[c]	184–186/ 245–249	390
Cortisone acetate (3-mono DNP[b])	B 2	Benz.-chlf.3:2[c]	240–242[d]	387
Δ^4-Cholestene-3-one	B 2	Hex.-benz.7:3	232[e] 239–241	390
Cholestane-3β-ol-6-one	A 1	Benz.-chlf.4:1	197[f]	368
Cholestane-3β-ol-6-one acetate	(A 1)	Hex.-benz.3:7	106	368
Δ^4-Cholestene-3β-ol-6-one acetate	B 2	Hex.-benz.1:9[c]	90–95	374
7-Ketocholesterol	A 1	Benz.-chlf.9:1	260–265	385
7-Ketocholesteryl acetate	B 1	Hex.-benz.1:1	218.5–221	385
$\Delta^{3,5}$-Cholestadiene-7-one	A 1	Hex.-benz.3:7	225–227	398
Methyl 12-ketocholanate	A 2	Hex.-benz.2:3	187–188.5	371
Methyl Δ^9-3α-hydroxy-12-ketocholenate	A 2	Benz.-chlf.4:1	197–198	385
Δ^5-Androstene-3β,17β-diol-16-one	A 1, A 2	Chloroform[c]	255	368
Δ^5-Androstene-3β,17β-diol-16-one diacetate	(A 1, A 2)	Benz.-chlf.9:1[c]	243–246	362–363
Androsterone	A 1	Benz.-chlf.9:1	233[a]	368
Androsterone acetate	(A 1)	Hex.-benz.2:3	196–201	367
Epiandrosterone	A 1	Benz.-chlf.4:1	215–216[g] / 190–195/ 224–225	372,[g] 368
Dehydroepiandrosterone acetate	B 2	Hex.-benz.1:4	231–234.5	367
Allopregnane-3β-ol-20-one	A 1	Benz.-chlf.4:1	269–271	368
Pregnane-3α-ol-20-one	A 1	Benz.-chlf.9:1	229[h]	367–368
Δ^5-Pregnene-3β-ol-20-one	A 1	Benz.-chlf.4:1	253–254[i]	368[j]
Δ^5-Pregnene-3β-ol-20-one acetate	B[k]	Hex.-benz.2:3	212–216[i]	368[i]
Progesterone (20-mono DNP[b])	A 1	Benzene	264–266	367

TABLE II—*Continued*

DINITROPHENYLHYDRAZONE OF	METHOD (see text)	ELUTED WITH	M.P., °C.	MAX IN $CHCl_3$, mμ
Pregnane-3α,12α-diol-20-one diacetate	B 2	Benzene	119–122	366
Allopregnane-3β,17α-diol-20-one	A 1	Benz.-chlf.1:1	291–293	362
Allopregnane-3β,17α-diol-20-one 3-monoacetate	(A 1)	Benz.-chlf. 9:1	239–241	362
$\Delta^{5,16}$-Pregnadiene-3β-ol-20-one	A 1	(Insoluble)	247–249	385
$\Delta^{5,16}$-Pregnadiene-3β-ol-20-one acetate	(A 1)	Hex.-benz.2:3	234–237	385
Δ^{4}-Androstene-3,17-dione (bis DNP[b])	A 1	Benzene	dec. 280	380
Adrenosterone (3,17-bis DNP[b])[i]	A 1	Benz.-chlf.4:1	dec. 280	375
Δ^{4}-Androstene-11β-ol-3,17-dione (bis DNP[b])	A 1	Benz.-chlf.4:1	191–193	377
Pregnane-3,20-dione (bis DNP[b])	A 1	Benzene	251–253[m] 265–267.5	368–369
Progesterone (bis DNP[b])	A 1	Benzene	282–283[n]	383,[o] 380
11-Ketoprogesterone (bis DNP[b])	A 1	Benzene	dec. 280	375
11β-Hydroxyprogesterone (bis DNP[b])	A 1	Benz.-chlf.4:1	276–278	380
17α-Hydroxyprogesterone (bis DNP[b])	A 1	Benz.-chlf.4:1	281–285	376–377
$\Delta^{4,16}$-Pregnadiene-3,20-dione (bis DNP[b])	A 1	Hex.-benz.1:4	193	386–387

[a] Johnston, *Science*, **106,** 91 (1947). [b] DNP= dinitrophenylhydrazone. [c] Acid-washed aluminum oxide Alcoa was used for chromatography. [d] Mattox and Kendall, *J. Biol. Chem.*, **188,** 287 (1951). [e] Rosenheim and Starling, *Chemistry & Industry*, **52,** 1056 (1933); Ralls, *J. Am. Chem. Soc.*, **55,** 2092 (1933). [f] Ralls, *J. Am. Chem. Soc.*, **55,** 2092 (1933) gives m.p. 257°. [g] Hilmer and Hess, *J. Am. Chem. Soc.*, **71,** 2947 (1949). [h] Marker and Lawson, *J. Am. Chem. Soc.*, **60,** 2438 (1938). [i] Reich, Sanfilippo, and Crane, *J. Biol. Chem.*, **198,** 713 (1952). [j] Djerassi and Ryan, *J. Am. Chem. Soc.*, **71,** 1000 (1949). [k] No excess dinitrophenylhydrazine was used in this experiment. [l] The 3-monodinitrophenylhydrazone (m.p. 254–256°) was described by Mason, Myers, and Kendall, *J. Biol. Chem.*, **116,** 267 (1936). [m] Lieberman, Dobriner, Hill, Fieser, and Rhoads, *J. Biol. Chem.*, **172,** 263 (1948). [n] Klein, Weiner, and Gordon, *Anal. Chem.*, **20,** 174 (1948). [o] Djerassi, *Anal. Chem.*, **20,** 880 (1948).

3β-ol-20-one (368 mμ) and allopregnane-3β,17α-diol-20-one (362 mμ), as well as the bisdinitrophenylhydrazones of progesterone (380 mμ) and 17α-hydroxyprogesterone (376 mμ). A similar effect was observed by introduction of a keto group in the 11-position. Both the bisdinitrophenylhydrazones of adrenosterone and 11-ketoprogesterone showed maxima at 375 mμ. The influence of the 11β-hydroxyl group seems to be more complex and requires further investigation.

Only a few dinitrophenylhydrazones were found soluble enough in carbon

disulfide for measurement of their infrared spectra. Δ^4-Cholestene-3-one dinitrophenylhydrazone showed bands at 2.99, 7.50, 7.66, 7.92, 8.17 (weak), and 8.82 μ which seem to be characteristic for the dinitrophenylhydrazine moiety. Since no bands were observed in the so-called keto region, it is possible to detect acetoxy and free keto groups, when they are present in a dinitrophenylhydrazone. Thus, desoxycorticosterone acetate 3-monodinitrophenylhydrazone exhibited bands at 5.68 μ (acetoxy group) and 5.77 μ (20-keto group), in addition to the bands given by Δ^4-cholestene-3-one dinitrophenylhydrazone. The acetoxy group could also be recognized by a band at 8.14 μ which was much stronger than that at 8.17 μ mentioned above. In chloroform solution all dinitrophenylhydrazones displayed bands at 2.98, 6.16, 6.25, 6.68, 7.49, 7.64, and 8.80 μ. Cortisone acetate 3-monodinitrophenylhydrazone gave additonal bands at 5.72, 5.77, and 5.84 μ characteristic for the 21-acetoxy group, the 20-keto group, and the 11-keto group respectively.

Recently a stable dinitrophenylhydrazine reagent containing phosphoric acid has been reported (13). When applied to Δ^5-pregnene-3β-ol-20-one, the corresponding dinitrophenylhydrazone was obtained in good yield. In the case of 17α-hydroxyprogesterone, however, partial dehydration took place. Consequently, in addition to the expected bisdinitrophenylhydrazone a considerable amount of $\Delta^{4,16}$-pregnadiene-3,20-dione bisdinitrophenylhydrazone was isolated from the reaction mixture.

EXPERIMENTAL[3]

One example of each method for removal of excess dinitrophenylhydrazine is described in this section.

Method A 1. 17α-Hydroxyprogesterone bisdinitrophenylhydrazone. To a solution of 11.3 mg. of 17α-hydroxyprogesterone in 1 cc. of abs. ethanol a solution of 25 mg. of 2,4-dinitrophenylhydrazine in 3 cc. of abs. ethanol and 6 drops of conc'd hydrochloric acid was added. The mixture was allowed to stand at room temperature overnight. After addition of 4 cc. of Benedict's reagent and 4 cc. of water, the suspension was heated on the water-bath for 10 minutes and extracted twice with chloroform. The solutions were washed once with water, dried, and evaporated. The residue was chromatographed on 3 g. of aluminum oxide Merck. The fractions eluted with benzene-chloroform 4:1 were recrystallized from chloroform-ethanol and gave red crystals, m.p. 282–285° (dec.); maxima at 259 mμ and 377 mμ (ϵ 20,563 and 33,928), minimum at 310 mμ (ϵ 6,041).

Anal. Calc'd for $C_{33}H_{38}N_8O_9$ C, 57.38; H, 5.55; N, 16.19.

Found: C, 57.59; H, 5.82; N, 15.91.

Method A 2. Δ^5-Androstene-3β,17β-diol-16-one dinitrophenylhydrazone. A solution of 10.0 mg. of steroid ketone in 0.5 cc. of abs. ethanol was mixed with 2.34 cc. of a solution containing 25.0 mg. of dinitrophenylhydrazine and 6 drops of conc'd hydrochloric acid in 3 cc. of abs. ethanol. The next day a solution of 150 mg. of potassium acetate in 1.5 cc. of abs. ethanol and two drops of acetone were added. After two hours standing at room temperature the mixture was diluted with chloroform and washed with water, very dilute

[3] All melting points were determined on a Kofler micro hot stage and are corrected. The ultraviolet spectra were taken in chloroform on a Beckman spectrophotometer Model DU, the infrared spectra on a Perkin-Elmer spectrophotometer Model 21. The microanalyses were carried out partly by Drs. Weiler & Strauss, Oxford, England and partly by Huffman Microanalytical Laboratories, Wheatridge, Colorado.

sodium carbonate solution, and again with water. The aqueous solutions were back extracted once with chloroform. Both chloroform solutions were dried, combined, and evaporated. The residue was chromatographed on 3 g. of acid-washed aluminum oxide Alcoa.[4] The fractions eluted with mixtures of hexane and benzene gave 16.0 mg. of acetone dinitrophenylhydrazone, corresponding to 13.3 mg. of dinitrophenylhydrazine. Since 19.5 mg. of the reagent was employed in the reaction, 6.2 mg. had been consumed (calculated 6.5 mg.). The fractions eluted with chloroform showed a maximum at 368–369 mμ and contained the steroid dinitrophenylhydrazone. They were dissolved in 1 cc. of pyridine and 0.5 cc. of acetic anhydride. After standing at room temperature overnight and following the addition of ice, the crystals were filtered, washed with water, dried, and chromatographed on 3 g. of acid-washed aluminum oxide Alcoa. The yellow diacetate was eluted with benzene-chloroform 9:1; it melted at 243–246.5° after two recrystallizations from chloroform-ethanol; maximum at 362–363 mμ.

Anal. Calc'd for $C_{29}H_{36}N_4O_8$: N, 9.86. Found: N, 9.80.

Method A 3. Methyl 3-ketocholanate dinitrophenylhydrazone. A solution of 15.0 mg. of methyl 3-ketocholanate in a few drops of methanol[5] was combined with 2.0 cc. of a solution containing 25.0 mg. of dinitrophenylhydrazine and 6 drops of conc'd hydrochloric acid in 3 cc. of methanol. After standing at room temperature overnight, a solution of 100 mg. of potassium acetate in 1 cc. of methanol and 1 drop of pyruvic acid was added. The mixture was allowed to stand for 1 hour, diluted with chloroform, and washed with water, two portions of 2 *N* carbonate solution, again with water, and dried. All aqueous solutions were back extracted once with chloroform. The combined chloroform solutions exhibited a maximum at 368 mμ. They were evaporated, and the residue was chromatographed on 3 g. of aluminum oxide Merck. The fractions eluted with hexane-benzene 2:3 yielded orange-yellow crystals from chloroform-methanol;[5] m.p. 145–150°.

Anal. Calc'd for $C_{31}H_{44}N_4O_8$: N, 9.85. Found: N, 9.96.

The alkaline wash solutions were acidified with dilute sulfuric acid and extracted four times with chloroform. The chloroform solutions were washed once with water, dried, and evaporated. The pyruvic acid dinitrophenylhydrazone thus obtained weighed 12.3 mg. This corresponds to 9.09 mg. of dinitrophenylhydrazine. Since 16.67 mg. was employed, 7.58 mg. was utilized (calculated 7.65 mg.). The pyruvic acid dinitrophenylhydrazone, after recrystallization from methanol, melted at 219–220° and exhibited a maximum at 347 mμ.[6]

Method B 1. 3-Ketocholanic acid dinitrophenylhydrazone. A suspension of 30.0 mg. of 3-ketocholanic acid and 30.0 mg. of dinitrophenylhydrazine in 18 cc. of chloroform and 3 cc. of glacial acetic acid was allowed to stand at room temperature for two days. To the clear yellow solution 4 drops of acetone were added. After standing for two hours the mixture was diluted with chloroform and washed with water, four 20-cc. portions of 2 *N* sodium carbonate solution, and again with water. The aqueous solutions were back extracted three times with chloroform, and the chloroform solutions were dried, combined, and evaporated. The residue weighed 21.7 mg. and was chromatographed on 3 g. of aluminum oxide Merck. The acetone dinitrophenylhydrazone eluted with hexane-benzene 3:2 weighed 17.0 mg. corresponding to 14.1 mg. of dinitrophenylhydrazine. The amount of dinitrophenylhydrazine utilized accounted for 15.9 mg. (calculated 15.9 mg.).

The alkaline solutions were acidified with 2 *N* sulfuric acid and extracted exhaustively with chloroform. The solutions were washed with water, dried, and evaporated. After two

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[4] This adsorbent was kindly supplied by Dr. H. B. MacPhillamy, Ciba Pharmaceutical Products, Summit, N. J.

[5] To avoid transesterification, methanol was used instead of ethanol.

[6] Braude and Jones, *J. Chem. Soc.*, 498 (1945), reported a maximum at 360 mμ (in chloroform). Dr. Braude informed us recently that with a fresh sample of pyruvic acid dinitrophenylhydrazone the following maxima were observed: 351 mμ (ε 24,000; photographic method) and 348 mμ (ε 23,500; photoelectric method).

recrystallizations from chloroform-ethanol yellow granules of m.p. 230–233° were obtained; maximum at 368 mμ.

Anal. Calc'd for $C_{30}H_{42}N_4O_8$: N, 10.10. Found: N, 9.96.

A sample was dissolved in 6 cc. of methanol, and 12 drops of conc'd hydrochloric acid were added. After standing at room temperature overnight the solution was evaporated *in vacuo*. The residue was taken up in chloroform and washed with a carbonate solution and water. After drying and evaporation, followed by chromatography and recrystallization from chloroform-methanol, methyl 3-ketocholanate dinitrophenylhydrazone, m.p. 145–150°, was obtained. It was identical with the preparation described above.

Method B 2. Δ⁴-Pregnene-11β,17α,20β,21-tetrol-3-one 20,21-diacetate dinitrophenylhydrazone. A suspension of 15.0 mg. of Reichstein's Compound E diacetate and 15.0 mg. of dinitrophenylhydrazine in 9 cc. of chloroform and 1.5 cc. of glacial acetic acid was kept at room temperature overnight. Then one drop of pyruvic acid was added to the clear red solution. After standing for one hour and dilution with chloroform, the mixture was washed with water, three 7.5-cc. portions of 2 *N* sodium carbonate solution, and again with water. The aqueous solutions were back extracted twice with chloroform. The chloroform solutions were dried, combined, and evaporated. The residue was chromatographed on 3 g. acid-washed aluminum oxide Alcoa. The fractions eluted with benzene-chloroform 3:2, after two recrystallizations from dilute ethanol, gave red granules with the double m.p. 184–186° and 245–249° (dec.); maximum at 390 mμ.

Anal. Calc'd for $C_{31}H_{40}N_4O_{10}$: N, 8.91. Found: N, 8.51.

The alkaline wash solutions were acidified with 2 *N* sulfuric acid and extracted three times with chloroform. The chloroform solutions were washed once with water, dried, and evaporated. The pyruvic acid dinitrophenylhydrazone weighed 10.9 mg. which corresponds to 8.05 mg. of dinitrophenylhydrazine. Thus 6.95 mg. of dinitrophenylhydrazine was utilized (calculated 6.63 mg.).

Johnson's method (13). 17α-Hydroxyprogesterone bisdinitrophenylhydrazone. A solution of 5 mg. of 17α-hydroxyprogesterone in 1.25 cc. of abs. ethanol was mixed with 0.25 cc. of Johnson's dinitrophenylhydrazine reagent. After standing overnight at room temperature, 2 cc. of 2 *N* sodium carbonate solution and 2 cc. of Benedict's reagent were added. The mixture was heated for 10 minutes on the water-bath and extracted twice with chloroform. The chloroform solutions were washed with water, dried, and evaporated. The residue was chromatographed on 3 g. aluminum oxide Merck. The fractions eluted with benzene weighed 5.5 mg. and were rechromatographed (see below). Benzene-chloroform 4:1 eluted 3.7 mg. of 17α-hydroxyprogesterone bisdinitrophenylhydrazone (maximum at 376–377 mμ), identical with the preparation described in the first experiment. The benzene fractions, after a second chromatography and recrystallization from chloroform-ethanol, gave a small amount of Δ⁴,¹⁶-pregnadiene-3,20-dione bisdinitrophenylhydrazone, m.p. 185–190°; maximum at 385 mμ. This substance was identical with the dinitrophenylhydrazone prepared from Δ⁴,¹⁶-pregnadiene-3,20-dione (m.p. 193°; maximum at 386–387 mμ).

The analytical data for several dinitrophenylhydrazones which have not heretofore been reported in the literature are listed in Table III.

2,4-Dinitrophenylhydrazine triacetate. A solution of 100 mg. of dinitrophenylhydrazine in 10 cc. of pyridine was mixed with 5 cc. of acetic anhydride. The solution turned light yellow. After standing overnight 90 cc. of water was added with cooling. The crystals were filtered, washed with water, dried, and recrystallized from a large volume of ethanol. The shining plates thus obtained softened at 195° and melted at 200–207°; maximum at 300 mμ.

Anal. Calc'd for $C_{12}H_{12}N_4O_7$: C, 44.45; H, 3.73; N, 17.28.
Found: C, 44.47; H, 3.80; N, 17.45.

The triacetate is only slightly soluble in benzene, chloroform, and ethanol. On chromatography with acid-washed aluminum oxide Alcoa it is eluted with benzene and mixtures of benzene and chloroform. The same derivative was obtained by acetylation of dinitrophenylhydrazine monoacetate (m.p. 199–200°; maximum at 325 mμ). After recrystallization from glacial acetic acid the triacetate softened at 193° and melted at 204–209°.

Anal. Calc'd for $C_{12}H_{12}N_4O_7$: C, 44.45; H, 3.73; N, 17.28.
Found: C, 44.50; H, 3.73; N, 17.63.

A sample of the triacetate (50 mg.) was refluxed for 1 hour in a mixture of 1 cc. of conc'd hydrochloric acid and 1 cc. of water. After addition of 2 g. of potassium acetate, the suspension was extracted four times with chloroform. The chloroform solutions were washed to neutrality, dried, and evaporated. The residue consisted of dinitrophenylhydrazine (maximum at 342 mμ).

TABLE III

ANALYTICAL DATA FOR DINITROPHENYLHYDRAZONES OF STEROID KETONES

DINITROPHENYLHYDRAZONE OF	NITROGEN	
	Calc'd	Found
3-Ketocholanic acid	10.10	9.96
Methyl 3-ketocholanate	9.85	9.96
Testosterone acetate	10.98	11.03
Methyl Δ^4-3,11-diketo-17α-hydroxyetiocholenate[a]	—	—
Progesterone (3-mono DNP[b])	11.33	11.31
Desoxycorticosterone acetate (3-mono DNP[b])	10.14	10.16
Δ^4-Pregene-11β,17α,20β,21-tetrol-3-one 20,21-diacetate	8.91	8.51
Cholestane-3β-ol-6-one acetate	8.97	9.00
Δ^4-Cholestene-3β-ol-6-one acetate	9.00	8.87
7-Ketocholesterol	9.65	9.55
7-Ketocholesteryl acetate	9.00	9.10
$\Delta^{3,5}$-Cholestadiene-7-one	9.96	10.37
Methyl 12-ketocholanate	9.85	10.00
Methyl Δ^9-3α-hydroxy-12-ketocholenate	9.62	9.65
Δ^5-Androstene-3β,17β-diol-16-one diacetate	9.86	9.80
Dehydroepiandrosterone acetate	10.98	10.91
Δ^5-Pregnene-3β-ol-20-one acetate	10.40	10.31
Pregnane-3α,12α-diol-20-one diacetate	9.36	9.50
Allopregnane-3β,17α-diol-20-one	10.89	11.19
Allopregnane-3β,17α-diol-20-one 3-monoacetate	10.07	10.05
Adrenosterone (3,17-bis DNP[b])	16.97	16.48
17α-Hydroxyprogesterone (bis DNP[b])	16.19	15.91
$\Delta^{4,16}$-Pregnadiene-3,20-dione (bis DNP[b])[c]	—	—

[a] Calc'd: C, 59.99; H, 5.97. Found: C, 59.98; H, 5.87. [b] DNP = dinitrophenylhydrazone. [c] Calc'd: C, 58.92; H, 5.40. Found: C, 59.45; H, 5.53.

2,4-Dinitrophenylhydrazine diacetate. Acetylation of 33.5 mg. of dinitrophenylhydrazine monoacetate with 2 cc. of pyridine and 0.02 cc. of acetic anhydride, followed by precipitation with water led to the formation of faintly yellow crystals which after recrystallization from ethanol melted over a wide range between 193° and 207°. The analysis indicated that this substance was a diacetate.

Anal. Calc'd for $C_{10}H_{10}N_4O_6$: N, 19.86. Found: N, 19.91.

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SUMMARY

Methods for the preparation and quantitative isolation of dinitrophenylhydrazones of steroid ketones have been described. By determining the excess dinitrophenylhydrazine as acetone dinitrophenylhydrazone or pyruvic acid dinitrophenylhydrazone the amount of reagent consumed by a given ketosteroid can be estimated. Several new dinitrophenylhydrazones have been characterized by their melting points, chromatographic behavior, and ultraviolet maxima. The infrared spectra of some dinitrophenylhydrazones have been discussed.

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REFERENCES

(1) STRAIN, *J. Am. Chem. Soc.*, **57**, 758 (1935).
(2) KLEIN, WEINER, AND GORDON, *Anal. Chem.*, **20**, 174 (1948).
(3) VEITCH AND MILONE, *J. Biol. Chem.*, **158**, 61 (1945).
(4) HILMER AND HESS, *Anal. Chem.*, **21**, 822 (1949).
(5) REICH, SANFILIPPO, AND CRANE, *J. Biol. Chem.*, **198**, 713 (1952).
(6) MATTOX AND KENDALL, *J. Biol. Chem.*, **188**, 287 (1951).
(7) MATTOX, *J. Am. Chem. Soc.*, **74**, 4340 (1952).
(8) JONES, WILKINSON, AND KERLOGUE, *J. Chem. Soc.*, 391 (1942).
(9) BRAUDE AND JONES, *J. Chem. Soc.*, 498 (1945).
(10) DJERASSI AND RYAN, *J. Am. Chem. Soc.*, **71**, 1000 (1949).
(11) DJERASSI, *J. Am. Chem. Soc.*, **71**, 1003 (1949).
(12) REICH, WALKER, AND COLLINS, *J. Org. Chem.*, **16**, 1753 (1951).
(13) JOHNSON, *J. Am. Chem. Soc.*, **73**, 5888 (1951).