[FROM THE WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY]

OPTICAL-ANALYTICAL STUDIES ON STEROIDS.

I. LIGHT ABSORPTION BETWEEN 230–500 mμ OF α,β-UNSATURATED AND 3,6-DIKETO STEROIDS IN ALKALINE ETHANOL

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For the elucidation of structural features of unknown steroids available in minute quantities only, infrared spectrometry has been developed into a most powerful tool. It is possible by its use to determine the types of functional grouping and the number of carbonyls present in the molecule (*cf.* 1, 2). However, their exact localization is as yet feasible only in a limited number of specific cases. Ultraviolet absorption (*cf.* 3) and optical rotation measurements (*cf.* 4) have proved of help in deciding between alternative structural possibilities. The determination of mobilities in paper-chromatographic systems (*inter al.* 5) can render valuable clues toward the establishment of a more accurate structure and, in combination with the concept of conformational analysis (6), the configuration of certain substituents may be deduced. In addition, oxidative degradations (*cf.* 7, 8, 9) followed by estimation of the liberated formaldehyde (*inter al.* 10) and acetaldehyde (11) as well as the Zimmermann chromogen (*inter al.* 12, 13) can be employed with advantage for the elucidation of side chain structure. The position of a ketone group may be more accurately defined by the ultraviolet spectrum of a derivative such as a phenylhydrazone (*inter al.* 3, 14, 15). The action of perchloric acid on certain substituted 2,4-dinitrophenylhydrazones should produce a bathochromic shift by extension of the conjugated system (*cf.* 16). The color formed after reaction of steroids with diphenylamine in acidic medium appears to depend on certain structural arrangements (17). A method has been recently devised for the recognition and estimation of Δ^4-3-ketosteroids by measuring the formation of intensely fluorescent substances resulting from alkaline treatment (18, 19). On the other hand, absorption spectra in concentrated sulfuric acid (*cf.* 20, 21), valuable as they are for the characterization of individual compounds, do not lend themselves for extensive interpretation of structural elements (*cf.* 22, 23).

In the papers presented in this series, an attempt is made to implement the methods enumerated, prompted by the specific need to determine structures of unknown Δ^4-3-ketosteroids isolated in milligram quantities from biological material. In this communication it is desired to report on the changes encountered in the intense absorption of a number of Δ^4-3-ketosteroids upon exposure to alkaline ethanol at 23 and 60°. Of particular interest were Δ^4-3-ketosteroids containing hydroxyl substituents at various positions and related substances with carbonyl functions. Alteration in spectral absorption of the color products has allowed correlation of certain structural features of these compounds. Some applications have been reported previously (24–27).* Paper II of this series pre-

* Incidentally, by this method the presence of a certain impurity was easily detected in a sample, although it had not been recognized in two paper-chromatographic systems.

sents the results of rate studies of formation of the "blue formazan" chromogen which may indicate the site of a substituent (26). Report III summarizes some observations on isolated olefinic centers of α,β-unsaturated ketonic steroids as related to absorption near 200 mμ (28).

METHOD

The source of the *steroids* is acknowledged below. Those compounds which are, throughout the Tables, designated by *p* were prepared by us following published procedures. Those samples designated by *q* were commercially available materials purified by direct crystallization, in certain cases preceded by chromatography. The steroids, before weighing, were dried at 0.1 mm. and 80° for 1 hr. The *reagent* was freshly prepared by mixing 94.0 ml. of 95% redistilled ethanol with 6.0 ml. of 10% aqueous tetramethylammonium hydroxide or 1.1 *N* aqueous potassium hydroxide. The solution thus had a normality of 0.066 with respect to hydroxide and a 90% ethanol content.

A quantity of steroid (between 50–70 μg.) to make an approx. 7×10^{-5} *M* solution was transferred to a stoppered silica cell of 10 mm. light path. The solvent used for the transfer was evaporated, at a temperature of no more than 35°, under a stream of nitrogen. To the dried sample, 3.0 ml. of alkaline reagent was added and the material readily dissolved. The absorbance, over a reaction blank, was then immediately recorded from 230 to 400 or 500 mμ by use of a Cary spectrophotometer (Model 11 MS). The region of 240 mμ, where Δ^4-3-ketosteroids absorb intensely, was reached 2–3 minutes after the steroids came in contact with the reagent. Further measurements were carried out at appropriate time intervals. When the reaction was conducted at elevated temperature, the cells were cooled under tap water to room temperature after their exposure. The absorptivity (ϵ) is expressed for concentrations in mole per liter.

In some instances, 0.20 ml. of 1 *N* aqueous hydrochloric acid was added after completion of the alkaline reaction and the spectrum of the solution, now at a *p*H between 7 and 3, was again recorded.

RESULTS AND DISCUSSION

A number of α,β-unsaturated steroids investigated were only slightly affected by the alkaline reagent under the described conditions (Table I). The group of Δ^4-3-ketosteroids in which a moderate decrease in absorbance near 240 mμ was observed, frequently gave a concomittant rise to a second less intense absorption near 380 mμ which became more distinct when the samples were heated to 60°. With 16α-hydroxyprogesterone (*# 12*), in addition, dehydration to 16-dehydroprogesterone appeared to have occurred, manifested by an increase in absorbance near 240 mμ within 4 hr. All of the six Δ^4-3-ketosteroids studied, with an unbonded oxygen at C-11, could be classed in the group mentioned (Table II). However, aldosterone (*# 21*) did not show the second band near 380 mμ; this is probably due to the fact that the 11β-hydroxyl group is largely tied up in a half acetal linkage with the 18-aldehyde function (*cf.* 29). Cross, *et al.* (30) noted a maximum near 375 mμ of unspecified intensity for cortisone acetate, progesterone, and testosterone, after heating these steroids in 1.1 *N* aqueous tetramethyl-

TABLE I

ABSORPTION MAXIMA IN mμ ($\epsilon \times 10^{-3}$) OF α,β-UNSATURATED KETOSTEROIDS IN ALKALINE ETHANOL AT 23°C.

	Compound	After 2-3 min.	After 24 hr.	After additional 1.5 hr. at 60°	Source
1	Δ⁴-Androstene-3,17-dione	241 (17.2)	241 (17.0)	241 (16.3) 377 (0.5)	*q*
2	Δ⁴-Androstene-17β-ol-3-one	241 (17.7)	242 (17.5)		*q*
3	3-Keto-Δ⁴-etienic acid	242 (17.2)	243 (15.6)	243 (14.4) 377 (0.6)	*p*
4	Δ⁴-Pregnen-21-ol-3,20-dione	242 (17.0)	242 (16.5)	243 (15.0) 377	*q*
5	Δ⁴-Cholesten-3-one	242 (17.1)	243 (16.4)		*d*
6	Δ¹,⁴-Androstadiene-3,17-dione	244 (16.5)	243 (16.6)	243 (16.5)	*n*
7	Δ⁴,⁶-Androstadiene-3,17-dione	284 (25.5)	284 (25.5)		*f*
8	7β-Hydroxy-Δ⁴-cholesten-3-one*	245 (15.6)	286 (25.9)	286 (24.2)	*p*
9	12α-Hydroxy-Δ⁴-androstene-3,17-dione	240 (17.0)	241 (16.6)		*o*
10	14-Hydroxy-Δ⁴-androstene-3,17-dione	238 (15.4)	243 (13.7) 377 (0.7)		*m*
11	16α-Hydroxy-Δ⁴-androstene-3,17-dione	241 (17.6)	242 (16.7)		*c*
12	16α-Hydroxy-Δ⁴-pregnene-3,20-dione†	242 (17.7)	242 (21.5) 375 (0.5)	241 (20.5) 375 (1.2)	*c*
13	Δ⁵-Cholesten-3β-ol-7-one acetate	235 (14.0)	239 (12.6) 279 (3.8)	239 (10.0) 279 (9.1)	*d*
14	Δ⁵,¹⁶-Pregnadien-3-ol-20-one	238 (9.9)	238 (4.8)		*q*

* This sample (*cf.* 32) showed a maximum at 243 mμ (ϵ 15.6 × 10³) in neutral abs. ethanol; $[\alpha]_D^{28}$ +84° ±3° (*c*, 0.95 in chloroform).

† This sample showed an absorption peak at 241 mμ (ϵ 17.8 × 10³) in neutral 95 per cent ethanol; the higher extinction coefficient in alkaline ethanol listed was reached before 4 hr. elapsed and afterwards decreased only slightly.

ammonium hydroxide solution at 70° for 35 minutes. Since this band was not present in dehydrocholic acid and some steroidal estrogens, they associated it with the Δ⁴-3-keto moiety. After conclusion of the present investigation, we learned of the successful development of a method for the quantitative estimation of Δ⁴-3-ketosteroids, based on the formation, by 0.3 *N* potassium *tert*-butoxide in *tert*-butyl alcohol, of an absorption band near 385 mμ in 1 hr. at room temperature (D. Abelson, private communication). The formation of this maximum appears to be greatly facilitated by the anhydrous reagent. Table III illustrates

TABLE II

ABSORPTIONS IN mμ ($\epsilon \times 10^{-3}$) OF 11-OXYGENATED Δ^4-3-KETOSTEROIDS IN ALKALINE ETHANOL AT 23°C.

Compound	After 2-3 min.	After 24 hr.		After additional 1.5 hr. at 60°			Source
	Max.	Max.	Max.	Max.	Min.	Max.	
15 Δ^4-Androstene-3,11,17-trione.	237 (16.7)	238 (14.9)	377 (1.6)	239 (10.0)	308 (0.6)	375 (3.9)	*p*
16 Δ^4-Pregnene-17α,21-diol-3,11,20-trione (cortisone)....	238 (17.0)	238 (13.1)	377 (0.9)	238 (11.1)	308 (0.7)	377 (1.5)	*q*
17 Δ^4-Androsten-11β-ol-3,17-dione.....	242 (17.4)	243 (18.0)	377 (0.9)				*p*
18 Δ^4-Pregnene-11β,17α,21-triol-3,20-dione.....	243 (17.5)	244 (15.3)	377	245 (14.0)		377 (0.9)	*q*
19 Δ^4-Androsten-11α-ol-3,17-dione.....	242 (17.9)	243 (16.3)	377 (0.8)				
20 Δ^4-Pregnen-11α-ol-3,20-dione..	242 (17.3)	241 (16.1)	377 (0.7)	243 (13.4)	308 (0.4)	377 (1.8)	*e*
21 Δ^4-Pregnene-11β,21-diol-18-oxo-3,20-dione (aldosterone).....	237 (17.8)	237 (17.2)	*I.* 285	237 (17.1)	*I.* 285		*h*

I = inflection.

that in Δ^4-3-ketosteroids in which the angular group at C-10 is actually (19-norsteroids) or potentially absent [eliminated by the action of the alkaline medium in 19-hydroxyl derivatives (25)], the second maximum was seen at 367 mμ rather than at 377 mμ as with the 10-methyl series. However, after acidification this second maximum shifted to approximately 310 mμ in the case of both series (*e.g.* *#12*, *15*, *20*, and *22*).

Most obvious, as was to be expected, were the changes caused by the alkaline reagent on the spectra of α,β-unsaturated ketosteroids having a hydroxyl substitutent in the vicinity of the unsaturated system. The *7β-hydroxy-Δ^4-3-keto* structure (*#8*) reacted in a characteristic manner: The absorption maximum at 245 mμ disappeared (it could no longer be clearly distinguished after 2 hr.), while simultaneously a new maximum at 286 mμ (first visible after 30 min.) developed. Since $\Delta^{4,6}$-cholestadien-3-one is reported (31) to absorb in the ultraviolet in neutral ethanol maximally at 285 mμ (ϵ 26.0 × 10^3), the absorbance changes in alkaline ethanol of *#8* represented the rate of dehydration of this compound. When compound *#8* was exposed to 0.03 *N* hydrochloric acid in 92% ethanol at 23°, the absorption peak at 245 mμ did not change over a period

TABLE III

ABSORPTION MAXIMA IN mμ ($\epsilon \times 10^{-3}$) OF 19-HYDROXYLATED AND 19-NOR-Δ^4-3-KETOSTEROIDS IN ALKALINE ETHANOL AT 23°C.

Compound	After 2–3 min.	After 24 hr.	After additional 1.5 hr. at 60°	Source
22 19-Nor-Δ^4-androstene-3,17-dione	240 (17.2)	241 (12.8) 367 (0.4)	242 (7.4) 367 (1.2)	*d*
23 19-Nor-Δ^4-pregnene-3,20-dione	241 (17.5)	242 (14.2)	242 (10.6) 367 (1.1)	*k*
24 19-Hydroxy-Δ^4-androstene-3,17-dione	243 (16.0)	242 (14.3) 367 (0.5)	241 (9.6) 367 (1.2)	*p*
25 19-Hydroxy-Δ^4-pregnene-3,20-dione	243 (16.0)	242 (14.4) 367 (0.3)	241 (9.6) 367 (1.2)	*a*
26 19-Hydroxy-3-keto-Δ^4-etienic acid	*br.* 245 (16.2)	244 (14.7)	*br.* 242 (12.4) 367 (1.1)	*a*

br. = broad.

TABLE IV

ABSORPTIONS IN mμ ($\epsilon \times 10^{-3}$) OF 2α-HYDROXYLATED Δ^4-3-KETOSTEROIDS[a] IN ALKALINE ETHANOL AT 23°C.

Exposure Time	Δ^4-Androstene-2α,17β-diol-3-one (*27*)			
	Max.	Inflection	Min.	Max.
Neutral	240 (15.8)			
2–3 min.	238 (14.9)			
1.0 hr.	234 (17.6)			354
4.0 hr.	233 (20.2)	254 (7.9)	290 (1.3)	355 (2.7)
24 hr.	233 (19.5)	254 (7.9)	290 (1.4)	355 (2.9)
	Δ^4-Pregnen-2α,ol-3,20-dione acetate (*28*)			
	Max.	Inflection	Min.	Max.
Neutral	240 (16.7)			
2–3 min.	238 (15.0)			
1.0 hr.	234 (19.0)			354
4.0 hr.	233 (20.4)	254 (7.8)	290 (1.2)	355 (2.6)
24 hr.	233 (19.8)	254 (7.8)	290 (1.2)	355 (2.7)
	Δ^4-Pregnene-2α,17α-21-triol-3,20-dione (*29*)			
	Max.	Inflection	Min.	Max.
2–3 min.	238 (15.6)			
1.0 hr.	234 (17.1)			354
4.0 hr.	233 (19.2)	254 (8.7)	290 (1.3)	355 (2.4)
24 hr.	233 (18.1)	254 (8.7)	290 (1.4)	355 (2.8)

[a] Source of these samples, *l*.

of 24 hr. and it lost only 5% of its intensity after additional heating at 60° for 1.5 hr. This behavior in an acidic medium may well prove to be a means of distinguishing Δ^4-7β-hydroxyl from the Δ^4-7α-hydroxyl derivatives, since Greenhalgh, *et al.* (32) observed a dehydration when hydrolyzing the 7α-tetrahydropyranyloxy-Δ^4-cholesten-3-one in very dilute ethanolic hydrocholoric acid at room temperature. Similarly, the data for Δ^5-*cholesten-3β-ol-7-one* acetate (*#13* would suggest that a partial conversion to $\Delta^{3,5}$-cholestadien-7-one had taken place; this dienone reportedly (33) has a peak at 280 mμ (ϵ 27 × 10^3) in neutral

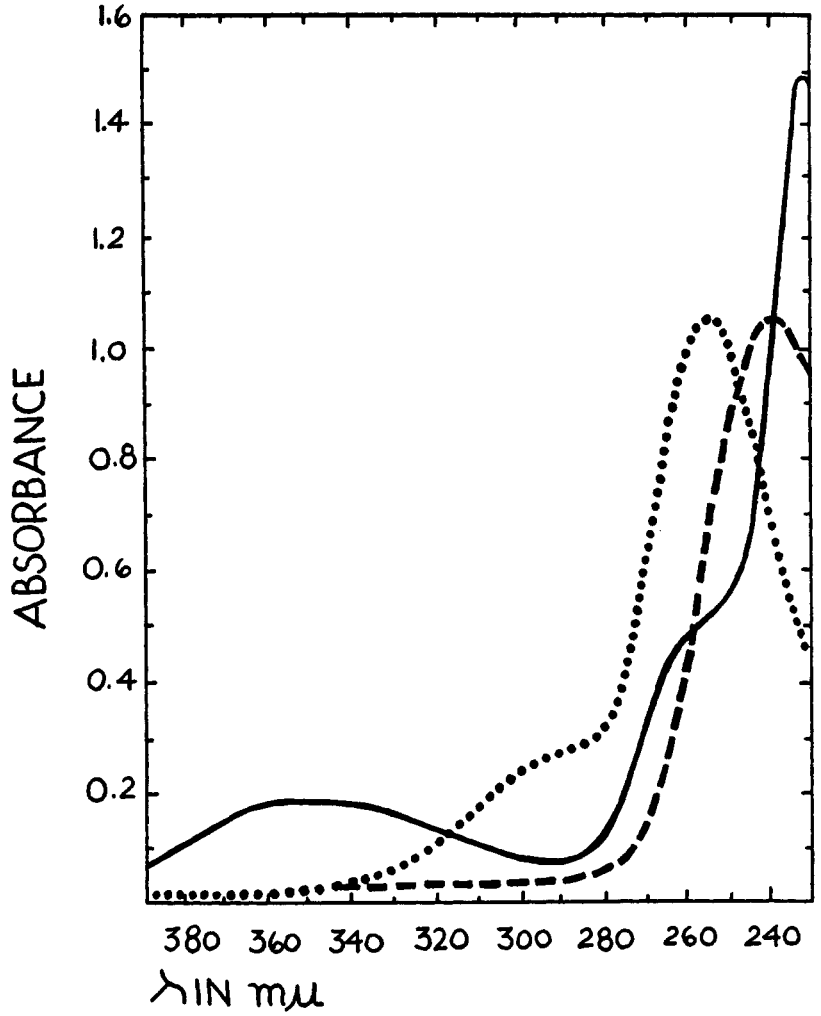

FIG. 1. SPECTRA OF 58.5 μg. OF Δ^4-ANDROSTENE-2α,17β-DIOL-3-ONE (*#27*) IN 3.0 ML. OF SOLVENT:- - - in 0.066 *N* tetramethylammonium hydroxide of 90% ethanol content, 2-3 minutes after solution; ——— in the alkaline solution after 4 hour of exposure; ••• same as latter conditions, however acidified with hydrochloric acid to *p*H 3 (ethanol content 85%).

ethanol. After acidification, the positions of these maxima at 280 mμ remained unchanged.

Three *2α-hydroxylated* Δ^4*-3-ketosteroids* with various side chains were studied. In all these cases the absorptivity near 240 mμ increased appreciably within the first 4 hr. of reaction; the integrated area of this band, however, remained approximately constant. The appearance of a typical shoulder near 255 mμ and a second maximum near 360 mμ led to an easily recognizable spectrum (Table IV and Fig. 1). The 2β-acetoxy-Δ^4-3-ketosteroids should show the same pattern, since their mild alkaline hydrolysis afforded the 2α-hydroxyl isomers (34). The profound change in the spectrum after acidification, which can be reverted after renewed addition of alkali, is noteworthy.

The allylic *6α and 6β-hydroxylated* Δ^4*-3-ketosteroids* were easily distinguished under the described conditions (Tables V and VI). The absorption maximum near 240 mμ of the former compounds (*# 33–35*) decreased to approximately half the intensity and shifted to 260 mμ within 24 hr. at 23° or 2 hr. at 60°. In the same period, a second maximum of somewhat lower intensity than the first one appeared near 380 mμ, the solution turning yellow. The 6β-hydroxylated steroids (*# 31* and *32*) did not produce this shift at room temperature and absorption near 380 mμ was less intense; at 60° the bathochromic shift occurred which, however, was delayed by approximately 1 hr. as compared to the 6α-hydroxylated derivatives. The saturated 3,6-diketone compounds (*# 36–39*), which do not absorb intensely in neutral ethanol, gradually gave rise to the two peaks which

TABLE V

ABSORPTIONS IN mμ ($\epsilon \times 10^{-3}$) OF 6-OXYGENATED STEROIDS IN ALKALINE ETHANOL AT 23°C.*

Exposure Time	3β-Hydroxy-Δ^4-6-keto-cholesten acetate (*30*)			6α-Hydroxy-Δ^4-3-keto derivatives of cholestene 6α-acetate (*33*)		
	Max.	Min.	Max.	Max.	Min.	Max.
2–3 min.	237 (5.5)			241 (16.3)		
4.0 hr.	243 (6.0)			245 (9.2)	315 (1.3)	377 (3.4)
10 hr.	243 (6.0)	320	380 (1.0)			
24 hr.	243 (6.1)	315 (0.8)	380 (2.2)	260 (8.7)	308 (2.0)	377 (6.5)
	6β-Hydroxy-Δ^4-3-keto derivatives of androsten-17-one (*31*)†			of androsten-17-one (*34*)‡		
2–3 min.	236 (14.2)			241 (16.4)		
1.0 hr.				242 (12.8)	312	377 (2.4)
4.0 hr.	238 (12.6)		377 (1.0)	246 (8.7)	310 (1.4)	377 (4.8)
10 hr.				258 (9.8)	308 (1.5)	377 (7.7)
24 hr.	240 (9.3)	308 (0.7)	377 (3.5)	258 (9.2)	308 (1.7)	377 (7.3)
	of pregnene-17α, 21-diol-20-one (*32*)			of pregnene-17α, 21-diol-20-one 6α, 21-diacetate (*35*)		
2–3 min.	237 (13.3)			237 (15.7)		
2.0 hr.						
4.0 hr.	238 (11.6)		377 (0.8)	246 (9.3)	323 (0.9)	377 (2.3)
10 hr.						
24 hr.	240 (8.5)	310 (0.9)	377 (2.2)	258 (7.3)	310 (1.6)	377 (5.0)

TABLE V—*Continued*

Exposure Time	3,6-Diketo derivatives of cholestane (*36*)§			Δ⁴-3,6-Diketo derivatives of cholestene (*42*)		
	Max.	Min.	Max.	Max.	Min.	Max.
2–3 min.				260 (11.3)	308 (1.9)	377 (9.3)
4.0 hr.	260 (4.7)		377 (3.3)	260 (10.9)	308 (1.6)	377 (8.7)
10 hr.	260 (8.2)	308 (1.9)	377 (6.6)			
24 hr.	260 (8.2)	308 (1.2)	377 (6.2)	260 (9.2)	308 (1.3)	377 (7.7)
	of androstan-17-one (*39*)			of androsten-17-one (*43*)¶		
2–3 min.				259 (10.8)	306 (1.6)	377 (9.1)
1.0 hr.	258 (3.9)	312	377 (2.7)			
4.0 hr.	258 (7.6)	308 (1.2)	377 (5.7)	259 (10.3)	306 (1.6)	377 (9.0)
10 hr.	258 (9.6)	308 (1.5)	377 (7.3)			
24 hr.	258 (9.0)	308 (1.7)	377 (6.6)	259 (10.1)	306 (1.6)	377 (8.4)
	of cholestane-4α,5α-diol (*40*)			Δ⁴-6-Keto-cholestene (*44*)		
2–3 min.		313 (0.7)	384 (2.2)	242 (7.9)		
2.0 hr.		308 (1.4)	377 (7.8)	242 (7.9)		380 (0.9)
4.0		308 (1.6)	377 (7.8)	244 (7.2)	315 (0.9)	380 (2.8)
10 hr.	*I*240	308 (1.7)	377 (7.4)	253 (6.3)	315 (1.1)	380 (3.6)
24 hr.	*I*240	308 (1.8)	377 (6.8)	257 (5.5)	310 (1.3)	380 (3.8)
	of 4-keto-cholestane (*41*)			Δ³,⁴-6-Keto-cholestadien (*45*)		
Neutral	275 (5.2)	298 (2.8)	341 (7.0)			314 (7.4)
2–3 min.	238 (3.2)	296 (0.6)	357 (4.8)		264 (1.6)	316 (6.9)
4.0 hr.	233 (5.5)	250 (5.2)			268 (2.8)	316 (6.2)
	258 (5.3)	296 (1.2)	357 (8.3)			
24 hr.	258 (4.7)	296 (1.1)	357 (7.7)	249 (6.3)	294 (3.3)	313 (3.8)
					360 (1.6)	405 (1.9)
+1.5 hr. (60°)				250 (8.8)	340 (1.0)	405 (2.4)

* Source of samples: *#30*, *44* and *45* from *g*; *#31* and *43* from *p*; *#32* from *e*; *#33* and *42* from *b*; *#34*, *35* and *39* from *a*; *#36* and *46* from *d*; *#37* and *38* from *f*; *#40*, *40a*, and *41* from *i*.—Compounds *#31*, *39* and *42* were further studied at the same alkalinity in aqueous 60 per cent ethanol and gave similar results, except that the bands near 260 mμ were shifted 2 mμ toward longer wave-lengths and the chromophore formation of compound *#39* was slower (the highest extinction values were reached after approximately 24 hours instead of 10 hours).

† The 6β-acetate (from *a*) showed essentially the same rate.

‡ The 6α-acetate (from *a*) showed essentially the same rate, except that the shift toward 241 mμ was attained only after 10 minutes; the absorbance maximum was located at 239.5 mμ after 2–3 minutes.

§ 3,6-Diketocholanic acid (*#37*) and 3,6-diketoallocholanic acid (*#38*) gave similar curves, the chromogen developed slower.

¶ The spectra (after 24 hr. exposure to the alkaline medium) were virtually the same when the solutions were diluted with 12 ml. of ethanol in a silica cell of 50 mm. light path.

I = Inflection.

reached their maximum in 10–14 hr. at room temperature. These compounds produced essentially the same absorptivity pattern as the corresponding 6α-hydroxyl derivatives after approximately 12 hr. at room temperature and 2–3 hr. at 60° and the 6β-hydroxy derivatives after 3–4 hr. at 60°. The spectral shift of

TABLE VI

ABSORPTIONS IN mμ (ε × 10^{-3}) OF 6-HYDROXYLATED Δ⁴-3-KETOSTEROIDS IN ALKALINE ETHANOL AT 60°C.

Exposure Time	Δ⁴-ANDROSTENE-3-17-DIONE					
	6β-Hydroxy derivative (*31*)			6α-Hydroxy derivative (*34*)		
	Max.	Min.	Max.	Max.	Min.	Max.
30 min.	238 (13.0)			243 (8.3)	310 (1.0)	377 (3.5)
1 hr.	239 (10.6)	320 (1.5)	377 (2.2)	257 (7.2)	308 (1.2)	377 (5.4)
2 hr.	240 (9.2)	308 (1.4)	377 (3.5)	258 (9.7)	308 (1.7)	377 (7.5)
3 hr.	258 (8.6)	308 (2.3)	377 (6.4)	258 (9.6)	308 (2.3)	377 (7.4)
4 hr.	258 (8.9)	308 (2.6)	377 (6.3)	258 (8.5)	308 (2.2)	377 (6.5)
	Δ⁴-PREGNENE-17α,21-DIOL-3,20-DIONE					
	6β-Hydroxy derivative (*32*)			6α-Hydroxy 6,21-diacetate (*35*)		
30 min.	238 (11.9)			240 (8.9)	325	377 (1.8)
1 hr.	238 (10.7)		377 (1.4)	245 (6.7)	320	377 (1.9)
2 hr.	240 (8.2)	315	377 (2.6)	254 (6.8)	312 (1.6)	377 (4.2)
3 hr.	253 (5.6)	313 (1.1)	377 (3.3)	258 (7.1)	310 (1.7)	377 (4.7)
4 hr.	258 (6.6)	310 (1.6)	377 (4.1)	258 (7.4)	310 (2.0)	377 (4.7)

3β-hydroxy-Δ⁴-6-keto-cholestene (*# 30*) occurred at a similar rate to that of the 6β-hydroxy-Δ⁴-3-keto derivatives. The behavior of these two 6-oxygenated substances was not unexpected since isomerization of such structures to the 5α-3,6-diketo derivatives in alkaline solution at elevated temperature were reported (35, 36, 36a). However, the more rapid shift of the Δ⁴-6α-hydroxyl compounds, which would indicate that such isomerization would occur more easily (*cf.* 26), was surprising in view of a report (36) that no isomerization was observed nder alkaline conditions when Δ⁴-pregnene-6α,21-diol-3,20-dione diacetate was hydrolyzed. The varied conditions might well explain the difference in result. Furthermore, the unsaturated Δ⁴-3,6-diketosteroids (*# 42* and *43*) turned yellow immediately after contact with alkaline ethanol and likewise exhibited absorption near 260 mμ and a second of approximately three-fourth its intensity near 380 mμ (Fig. 2). After acidification, these peaks shifted in all cases to 253 and 315 mμ, respectively.

Exposure of compound *# 43* to 0.07 *N* hydrochloric acid in 90% ethanol for 4 hr. at room temperature brought about the appearance of a second maximum at 315 mμ (ε 2.9 × 10^3); the maximum at 252 mμ decreased only slightly to an absorptivity of 10.3 × 10^3. Under the same acidic conditions, the spectrum of compound *# 34* was not significantly influenced over a period of 24 hr. However isomerization of 6α- and 6β-hydroxy-Δ⁴-3-keto compounds to the saturated derivatives was reported to occur when the compounds were refluxed in alcoholic solutions of 0.1–0.6 *N* hydrochloric acid (41, 36a, see also 36). When microgram quantities of the Δ⁴-3,6-diketosteroids (*# 42* and *43*) were stored for about a month at 4° in a stoppered glass tube in dry state or in neutral ethanolic solution, the appearance of a second maximum near 315 mμ was also observed.

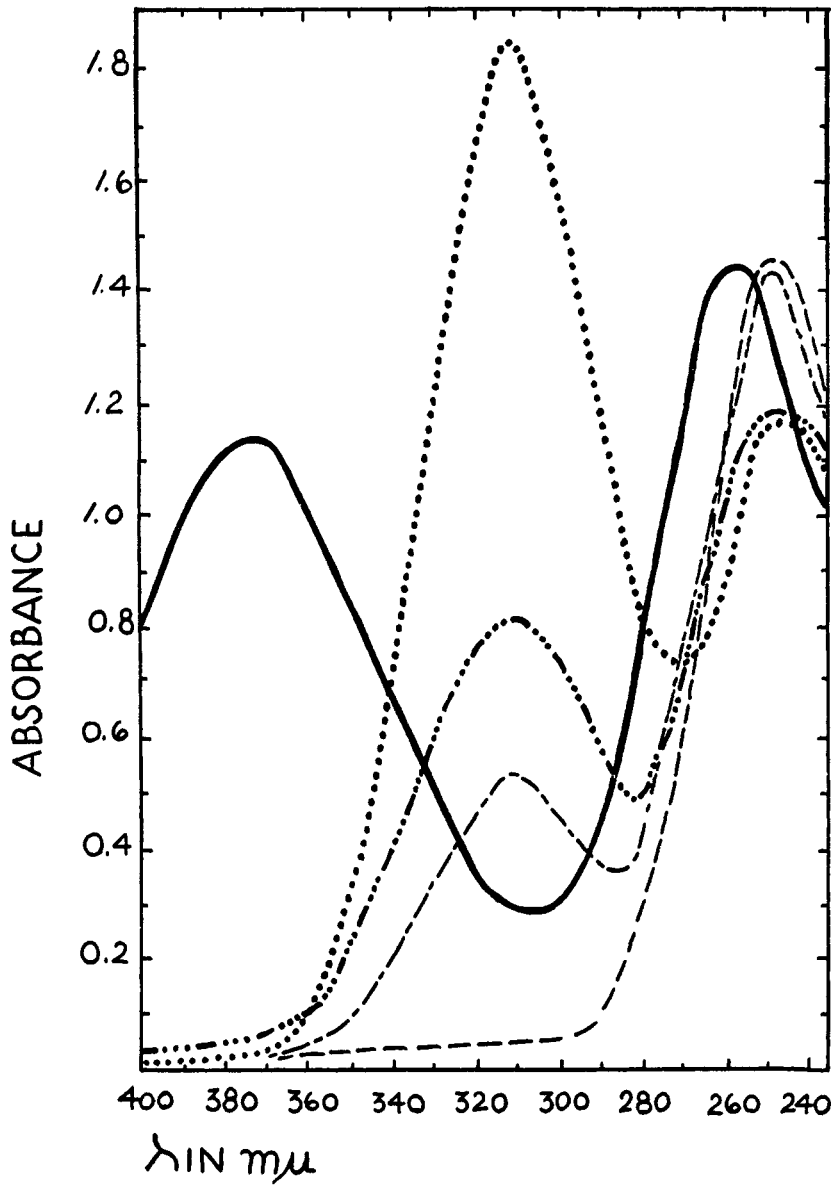

FIG. 2. SPECTRA OF 115.0 μg. OF Δ^4-ANDROSTENE-3,6,17-TRIONE (*#43*) IN 3.0 ML. OF SOLVENT: - - - in 95% ethanol; ——— in 0.066 *N* potassium hydroxide of 90% ethanol content (after 2–3 minutes); ••• 2 minutes after addition of the potassium hydroxide solution (2.8 ml.) acidified with 1 *N* hydrochloric acid (0.20 ml.) to *p*H 6.7 (ethanol content 85%); —·— acidified to *p*H 2.7; — ••• — same as latter conditions, steroid however exposed to the alkaline solution at 23° for 24 hours before acidification.

The action of alkali on 3,6-oxygenated steroids was noted previously. Windaus (37) found that 3,6-cholestanedione was not soluble in aqueous alkali. However, it dissolved in ethanolic potassium hydroxide producing a strongly yellow solution from which it was precipitated by the addition of large volumes of water, mostly as the unchanged material. Furthermore, Windaus (38) observed that solutions of aqueous potassium hydroxide had to have a strength of at least 20% in order to extract Δ^4-cholestene-3,6-dione from its ethereal solution. The freshly formed, only slightly soluble potassium salt (*cf.* 39) was easily decomposed to the free compound by addition of water. However, when the dried salt was stored for some days under a vacuum, it had undergone a rearrangement to the salt of a carboxylic acid of unknown nature. Ross (39) achieved total hydrolysis of the two dienol dibenzoates of Δ^4-cholestene-3,6-dione when the substances were refluxed with an excess of $N/10$ alcoholic potassium hydroxide for 1 hr. However, in contrast to the 3,6-dibenzoyloxy-$\Delta^{2,4,6}$-cholestatriene, no Δ^4-cholestene-3,6-dione was obtained from the experiment with the 3,6-dibenzoyloxy-$\Delta^{3,5,7}$-cholestatriene. Heard and Sobel (40) noted the occurrence of a shift of the main resonance band in the ultraviolet from 251.5 mμ (ϵ 10.6 $\times$ 10^3) to 259 mμ (ϵ 10.0 $\times$ 10^3) when the spectrum of Δ^4-cholestene-3,6-dione was examined in the presence of alkali rather than in neutral ethanol; with Δ^4-cholesten-3-one no such shift was seen. Fieser (41) showed again the formation of various enol anions, all with ultraviolet absorptions in the region of 260 mμ (ϵ $\sim$5 $\times$ 10^3), when hexane solutions of cholestane-3,6-dione, Δ^4-cholestene-3,6-dione, Δ^4-cholesten-6β-ol-3-one, and Δ^4-cholesten-3β-ol-6-one were shaken with 35% potassium hydroxide solution in aqueous methanol. Finally, it might be pertinent to add to this survey an observation of Greenhalgh, *et al.* (32). These workers found that Δ^5-cholestene-3,7-dione, which absorbs in ethanolic solution maximally at 320 mμ and is probably present as the $\Delta^{3,5}$-dien-7-one 3-enolate, produced also a bathochromic shift in its spectrum of approximately 70 mμ when alkali was added to the solution.

It is worthy of note that after exposure to alkaline ethanol a second absorption peak near 380 mμ was common to a number of different 6-ketosteroids. This band invariably shifted to approximately 315 mμ after acidification, but reverted to the previous absorptivity pattern after renewed addition of alkali. This typical spectrum was exhibited, as mentioned before, by the Δ^4-3,6-diketosteroids (**42* and *43*) *immediately* after contact with alkaline ethanol. Interestingly, the saturated 3,6-diketo derivatives also produced the same absorption pattern; however, approximately 10 hr. at room temperature were required until maximal intensity was reached. Again, approximately 10 hr. elapsed until the heteroannular Δ^4-6-ketocholestene (**44*) attained maximal absorptivity (at 380 mμ). On the other hand, 4α(or β),5α-dihydroxy-3,6-diketocholestane (**40* and *40a*) developed maximal absorption (at 377 mμ) already within 2 hr. (*cf.* 42). The latter two were the only compounds of the series with a single peak which shifted to 317 mμ after acidification. As exemplified by Δ^4-androstene-3,6,17-trione (Fig. 2), it can be seen that the intensities of the two bands, especially that near 315 mμ, are highly sensitive to the *p*H of the solution after acidification. The band

near 315 mμ reached maximal intensity when neutrality was approached. 6-Ketocholestanol acetate (*#46*) did not develop intense ultraviolet absorption in alkaline solution.

Finally, the action of alkali on two further substances is listed in Table V. $\Delta^{2,4}$-6-Ketocholestadien (*#45*), after alkaline treatment at 60°, had two maxima, at 250 and 405 mμ, which shifted after acidification to 239 and 327 mμ, respectively. In contrast, 3,4,6-triketocholestane (*#41*) produced, after alkaline treatment and acidification, a similar spectrum as it originally had exhibited in neutral ethanol (*cf.* 42), although during exposure to alkaline solution significant changes were observed. This indicates that the reaction products, which are probably enolic in nature, are formed at a relatively slow rate and that their formation is reversible.

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SUMMARY

The absorption spectra in alkaline medium of a number of Δ^4-3-ketosteroids and their hydroxylated derivatives have been studied. Measurements were conducted in 0.066 *N* alkali in 90% ethanol at 23° and/or 60° over varying periods of time. Typical spectral shifts were observed which make it possible to use these data as a microanalytical tool for the characterization of various substituted Δ^4-3-ketosteroids. In addition, the spectral changes of a number of 6-oxygenated steroids are reported.

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