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Indirect Analysis of Corticosteroids

3. THE DETERMINATION OF STEROIDAL DIHYDROXYACETONES*

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Earlier papers of this series (Appleby, Gibson, Norymberski & Stubbs, 1955; Appleby & Norymberski, 1955) introduced two analytical methods, each of which involved a sequence of specific reactions designed to transform an accurately defined group of corticosteroids into 17-oxosteroids and the measurement of the latter as chromogens in the Zimmermann reaction. The same general principle has now been employed to develop a further method of steroid analysis. It is based on the observation (Norymberski, 1956) that steroidal dihydroxyacetones (17 α :21-dihydroxy-20-oxopregnanes) undergo hydrogenolysis at the C₁₇-O bond when treated with zinc powder in boiling aqueous acetic acid; so, for instance, cortisone (I) was converted into 11-dehydrocorticosterone (II). Consequent upon the removal of the 17-hydroxyl group the dihydroxyacetones lose their 17-ketogenic property (convertibility into 17-oxosteroids on oxidation with bis-

muthate; see Brooks & Norymberski, 1953). On the other hand, it was expected that 17:20-diols and 17:20:21-triols, i.e. the two other structural types of the 17-ketogenic group, will resist the action of zinc and hence that it should be possible to measure them selectively as 'zinc-resistant 17-ketogenic steroids'. The proposed analytical procedure was briefly outlined in a preliminary communication (Norymberski & Stubbs, 1955).

The preparative experiments referred to above were performed with only a moderate excess of zinc (10:1, w/w). When cortisone was reduced under conditions considered more suitable for analytical purposes, wherein a much larger excess of zinc (10 000:1, w/w) was employed, it was found that after 1 hr. reduction with zinc, and following oxidation with bismuthate, the amount of Zimmermann chromogens was decreased to a constant level. The nature of the residual chromogenic contribution, whose magnitude corresponded to about 20 % of the quantity expected if the cortisone had not been

* Part 2: Appleby & Norymberski (1955).

affected, was not immediately apparent. It could not derive from unchanged cortisone since the reduction product gave no colour with phenylhydrazine in sulphuric acid (Porter & Silber, 1950). Neither could it derive from 11-dehydrocorticosterone (II), since this compound would have been converted by bismuthate into an aetio-acid and the latter removed by the alkali wash preceding the Zimmermann reaction. It was therefore suspected that under the reaction conditions employed cortisone underwent more profound structural changes than in the preparative experiments. This view was confirmed by experiments in which cortisone was treated with various amounts of zinc (100-fold to 10 000-fold, w/w): it was found that (i) at all ratios of the reactants the reaction products were practically non-chromogenic in the Porter-Silber test, (ii) the extinction at 238 m μ . fell rapidly with increasing amounts of zinc, and (iii) the amount of formaldehyde released on oxidation with bismuthate fell slowly with increasing amounts of zinc (see Fig. 1). These results are interpreted on the assumption that the products formed at low ratios of reactants are intermediates in the reactions occurring at higher ratios. The initial step is then the removal of the 17-hydroxyl group, since it occurs with a tenfold excess of the reagent; the results of the Porter-Silber test are therefore taken as an indication of the extent of the reaction at C₍₁₇₎. The disappearance of specific absorption at 238 m μ . obviously indicates the reduction of the 4-en-3-one system; the reduction product gave a yellow colour with tetranitromethane and a negative

test with trichloroacetic acid (Rosenheim, 1929), it must therefore contain an olefinic bond but no hydroxyl group in the allylic position. Finally, the formaldehydogenic property is considered to be associated with the presence of the 21-hydroxyl group.

On the basis of these observations the reaction of cortisone with a large excess of zinc is tentatively formulated as follows. Reductive removal of the 17-hydroxyl group converts cortisone (I) into 11-dehydrocorticosterone (II); this is followed by reduction of the 4-en-3-one grouping, leading to a 21-hydroxypregnene-11:20-dione (III), and finally removal of the 21-hydroxyl gives a pregnene-11:20-dione (IV). The properties expected from compounds (I)–(IV) in the analytical tests employed (see Table 1) provide a satisfactory rationalization of the experimental findings; in particular, the formulation of the end product as a 20-ketone unsubstituted at C₍₂₁₎ explains the residual chromogenic contribution to the Zimmermann reaction.

Further confirmation of the proposed reaction sequence was obtained by treating tetrahydrocortisone (V) with a 500-fold excess of zinc. After acetylation three compounds were isolated: 3 α :21-diacetoxypregnane-11:20-dione (VIb), which was the

Fig. 1. Reaction of cortisone with zinc at various ratios of the reactants. O, Reaction at C₍₃₎ based on extinction at 238 m μ .; Δ , reaction at C₍₁₇₎ based on results of the Porter-Silber reaction; $\square$, reaction at C₍₂₁₎ based on release of formaldehyde with bismuthate. Filled-in points are approximate values derived from several preparative experiments.

Dotted lines in formulae (III) and (IV) denote two likely positions of the double bond.

Table 1. Summary of properties of cortisone (I) and its transformation products after reduction with zinc

Test	Compound			
	I	II	III	IV
Porter-Silber reaction	+	-	-	-
17-Ketogenic property	+	-	-	($\pm$)*
Specific absorption at 238 m μ .	+	+	-	-
Release of formaldehyde	+	+	+	-
Tetranitromethane	-	-	+	+

* Weakly chromogenic in the Zimmermann reaction and resistant to bismuthate.

Table 2. *Summary of reactions of model compounds with zinc**

Side chain	Compound	Zinc/steroid (w/w)	Side chain of product	Evidence
$\begin{array}{c} \text{CH}_2.\text{OH} \\ \\ \text{CO} \\ \\ \text{C}..\text{OH} \\ / \end{array}$	Cortisone	10:1	$\begin{array}{c} >\text{CH}.\text{CO}.\text{CH}_2.\text{OH} \\ >\text{CH}.\text{CO}.\text{CH}_3 \\ >\text{C}(\text{OH}).\text{CO}.\text{CH}_3 \end{array}$	Isolation of products†
	Cortisol			
	Tetrahydrocortisone			
$\begin{array}{c} \text{CH}_2.\text{OH} \\ \\ \text{CO} \\ \\ \text{C}..\text{OH} \\ / \end{array}$	Tetrahydrocortisone	500:1	$\begin{array}{c} >\text{CH}.\text{CO}.\text{CH}_2.\text{OH} \\ >\text{CH}.\text{CO}.\text{CH}_3 \\ >\text{C}(\text{OH}).\text{CO}.\text{CH}_3 \end{array}$	Isolation of products†
	Cortisone	10 000:1	$\begin{array}{c} >\text{CH}.\text{CO}.\text{CH}_3 \\ [>\text{C}(\text{OH}).\text{CO}.\text{CH}_3] \\ (?) \end{array}$	Decreased value as 17-KGS; negative Porter-Silber test; not formaldehydogenic†
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CO} \\ \\ \text{C}..\text{OH} \\ / \end{array}$	3 α :17 α -Dihydroxypregnane-11:20-dione	10:1	Unchanged	Isolation of starting material†
		10 000:1	Unchanged	No significant change in assays of 17-KS, 17-KGS, and 17-OH CS†
$\begin{array}{c} \text{CH}_2.\text{OH} \\ \\ \text{CH}.\text{OH} \\ \\ \text{C}..\text{OH} \\ / \end{array}$	17 α :20 β :21-Trihydroxy-pregn-4-en-3-one	10 000:1	Unchanged	17-Ketogenic property retained†
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}.\text{OH} \\ \\ \text{C}..\text{OH} \\ / \end{array}$	3 α :17 α :20 β -Trihydroxy-pregnan-11-one	10 000:1	Unchanged	17-Ketogenic property retained†
$\begin{array}{c} \text{O} \\ \\ \text{C} \\ / \end{array}$	Dehydroepiandrosterone	10 000:1	Unchanged	Chromogenic value in the Zimmermann reaction retained†
$\begin{array}{c} \text{CH}_3.\text{OAc} \\ \\ \text{CO} \\ \\ \text{C}..\text{OH} \\ / \end{array}$	Cortisone acetate	10:1	Unchanged	Isolation of starting material†
		10 000:1	$>\text{C}(\text{OH}).\text{CO}.\text{CH}_3 \\ (?)$	Negative Porter-Silber test† Increased value as 17-KS; no significant change as 17-KGS and 17-OH CS†
	Tetrahydrocortisone acetate	10 000:1		

* See also Table 4.

† Norymberski (1956).

‡ This paper.

cortisone acetate (by mouth) or a preparation of the corticotrophic hormone (by intramuscular injection), and (iv) two cases of the adrenogenital syndrome. Within the normal group the ratio of SDHA to 17-KGS varied between 20 and 60 %. At higher excretion levels caused by the administration of cortisone acetate or corticotrophin the range of the same ratio was raised to 40–80 %, in pregnancy it was lowered to 10–30 %, and in the adrenogenital syndrome the ratio was less than 10 %.

The average normal excretion values of SDHA were (in mg.-equiv. of dehydroepiandrosterone/24 hr.): 4.9 (range 4.0–6.4) for men, and 3.6 (range 2.0–5.8) for women. Sandberg, Nelson, Glenn,

Tyler & Samuels (1953) found for glucuronide-bound dihydroxyacetones, measured as Porter-Silber chromogens (Glenn & Samuels, 1953), the following average normal excretion values (in mg.-equiv. of cortisol/24 hr.): 5.3 (range 3.3–9.3) for men and 3.2 (range 2.1–5.8) for women. In view of the profoundly different techniques employed in the two analytical methods, agreement between the results must be treated with caution.

EXPERIMENTAL

Melting points were determined on a Kofler stage. Rotations were measured in CHCl_3 (at 15–20°) and ultraviolet absorption spectra in ethanol. Analysis was by Weiler and

Strauss (Oxford). The Zimmermann reaction was performed according to Zygmuntowicz, Wood, Christo & Talbot (1951) and colorimetric readings were taken at 430 and 520 $m\mu$. and corrected (Medical Research Council, 1951). Chromogens with phenylhydrazine and sulphuric acid were determined essentially by the method of Porter & Silber (1950), the reaction being performed overnight at room temperature and the extinction measured at 430 $m\mu$. Formaldehydogenic steroids were determined by the method of Brooks & Norymbski (1953). Reductions with borohydride were carried out as previously described (Appleby *et al.* 1955), except that potassium borohydride was used instead of the sodium salt.

Preparative treatment of tetrahydrocortisone (V) with zinc powder

Tetrahydrocortisone (100 mg.) in aqueous acetic acid (50%, v/v; 100 ml.) was treated with Zn powder (50 g.; AnalaR) for 1.5 hr. at reflux. After cooling, the Zn was filtered off, refluxed with ethanol and filtered off again. Ice and then 3*N*-NaOH (200 ml.) were added and the mixture was extracted with ether in the usual manner. The crude product (80 mg.) was acetylated with acetic anhydride in pyridine at room temperature. The product was chromatographed on neutral alumina (8 g.) by a gradient-elution technique (see Appleby & Norymbski, 1955) with the solvent systems benzene–light petroleum (1:1)/benzene/benzene–ethyl acetate (98:2); sixty 4 ml. fractions were collected.

Fractions 6–9 (m.p. range 110–131°; 21.5 mg.) gave from acetone–hexane heavy prisms, m.p. 130–131°, $[\alpha]_D + 131^\circ$ (c, 1.25). (Found: C, 73.8; H, 9.45. Calc. for $C_{23}H_{34}O_4$: C, 73.8; H, 9.15%). The compound was identified as 3 α -acetoxy-pregnane-11:20-dione (VIIb) by its unchanged m.p. on admixture with an authentic specimen. Euw, Lardon & Reichstein (1944) reported for the compound m.p. 132–133°, $[\alpha]_D + 122^\circ$ (in acetone).

Fractions 13–25 (m.p. range 100–110°; 29.5 mg.) gave from acetone–hexane small plates, m.p. 107–109°, $[\alpha]_D + 123^\circ$ (c, 1.40). The mixed m.p. with authentic 3 α :21-diacetoxy-pregnane-11:20-dione (VIb; Norymbski, 1956) was unchanged.

Fractions 39–43 (m.p. range 193–202°; 6 mg.) gave from acetone–hexane prisms, m.p. 203–206°. The compound was identified as 3 α -acetoxy-17 α -hydroxypregnane-11:20-dione (VIIIb) by comparison of m.p. and infrared spectrum with those of an authentic specimen.

ANALYTICAL

Symbols and definitions

17-KS. 17-Oxosteroids in terms of dehydroepiandrosterone.

Total 17-KS. 17-KS found after treatment with bismuthate.

Zn/total 17-KS. 17-KS found after treatment with Zn and then with bismuthate.

17-KGS (17-ketogenic steroids). Total 17-KS – 17-KS.

Zn/17-KGS (zinc-resistant 17-KGS). Zn/total 17-KS – 17-KS.

SDHA (steroidal dihydroxyacetones). 17-KGS – Zn/17-KGS = total 17-KS – Zn/total 17-KS.

General procedure for treatment with zinc

A solution of the analytical sample (0.1–0.3 mg., unless otherwise stated) in aqueous acetic acid (50%, v/v; 10.0 ml.) is refluxed with Zn powder (1.0 g.; AnalaR) for 1 hr. (The reaction is conveniently performed in test tubes (6 in. $\times$ $\frac{1}{2}$ in.) fitted with cold-finger condensers and placed in an aluminium heating block maintained at 120–140°.) The reaction mixture is rapidly cooled to room temperature and further treated in one of the two following ways.

(i) *For the determination of Zn-resistant total 17-KS.* A sample (4.0 ml.) of the supernatant is shaken with $NaBiO_3$ (0.5 g.; AnalaR) for 0.5 hr. and worked up by reducing surplus reagent with $Na_2S_2O_5$ and extracting in the usual manner (see Gibson & Norymbski, 1954; Appleby *et al.* 1955). Samples of the washed extract of suitable size are taken for the Zimmermann reaction.

(ii) *For other analytical tests.* A sample (8.0 ml.) of the supernatant is treated with 3*N*-NaOH (4.0 ml.) and then extracted with ethylene dichloride (25.0 ml.); the extract is washed successively with water (5 ml.) and 3*N*-NaOH (5 ml.) and filtered through paper. Suitable portions of the filtrate are taken for the tests employed.

Variation of reaction time. Samples (100 μ g. each) of cortisone were treated by the general procedure (i) except that the time of reaction with Zn was varied between 10 and 90 min. In control experiments addition of Zn was omitted. Each experiment was duplicated. The results (mean values) are in Table 3.

Variation of reactant ratio. Samples of cortisone (0.1–10.0 mg.) were treated by the general procedure (ii). Samples of the final extract, equivalent to 22.4–64.0 μ g. of starting material, were taken for the measurement of (a) the extinction at 238 $m\mu$, (b) Porter–Silber chromogens, and (c) formaldehyde, released by bismuthate, as chromogen with chromotropic acid. Each measurement was corrected by subtraction of a value found for the corresponding process blank (without addition of cortisone). The results are presented in Fig. 1.

Tetranitromethane gave a yellow colour with products obtained at all concentrations of cortisone; no colour was produced with trichloroacetic acid in $CHCl_3$.

Variation of substrate. Samples of steroids listed in Table 4 were treated by the general procedure (i) or (ii),

Table 3. *Effect of zinc treatment on the 17-ketogenic property of cortisone*

Time at reflux (min.)	Recovery of cortisone*	
	As Zn/17-KGS (%)	As 17-KGS† (%)
0	—	79
10	46	—
20	30	79
30	29	77
45	21	81
60	20	75
90	18	71

* Recoveries calculated on the basis of quantitative conversion into androst-4-ene-3:11:17-trione and that compound's colorimetric equivalence to 0.97 mole of dehydroepiandrosterone (see Brooks & Norymbski, 1953).

† Controls without addition of zinc.

whichever applicable. In control experiments the addition of Zn was omitted. The products were submitted to one or more of the following analytical tests: (a) measurement of extinction at maximum absorption of starting material (237–242 m μ .); (b) measurement of Porter–Silber chromogens; (c) measurement of Zimmermann chromogens; (d) as (c) after treatment with bismuthate; and (e) as (d) after reduction with borohydride. All experiments except those under (a) were duplicated. The results (Table 4) are expressed in proportion to values found for corresponding control samples.

The reaction product obtained from cortisol, and to a lesser extent that obtained from its acetate, exhibited with the Porter–Silber reagent a strong absorption at 430 m μ . This observation cannot, however, indicate that the product is largely composed of a compound with an intact dihydroxyacetone grouping, since (i) its 17-KGS value was only 19% of that of cortisol (see Table 4), (ii) its absorption (with the Porter–Silber reagent) between 320 and 500 m μ . differed significantly from that of cortisol (see Fig. 3), and (iii) already with a tenfold excess of Zn cortisol was converted into corticosterone in high yield (Norymberski, 1956). It is therefore concluded that, under the conditions here employed, cortisol is transformed into a compound which, although a Porter–Silber chromogen, does not contain the dihydroxyacetone grouping.

Treatment of urine specimens with zinc. All specimens were from 24 hr. collections. Each was diluted with water to 2.0 l. and stored at 4°.

General procedure. A mixture of the urine (3.0 ml.), acetic acid (3.0 ml.), and Zn powder (0.6 g.; AnalaR) is kept at reflux for 1 hr. After cooling to room temperature a sample (4.0 ml.) of the supernatant is treated first with NaBiO₃ and then with HCl exactly as previously described for the determination of total 17-KS (Gibson & Norymberski,

1954). The total 17-KS and 17-KS are determined concurrently.

Variation of reaction time. Two urine specimens were treated by the general procedure except that the time of reaction with Zn was varied between 30 and 180 min. 17-KS and total 17-KS were determined in duplicate: the results are in Table 5. With one of the specimens control experiments were carried out in which the addition of Zn was omitted: it was found that boiling for up to 90 min. in the presence of acetic acid had no effect on the total 17-KS values of the urine.

Fig. 3. Chromogenic (Porter–Silber) properties of cortisol and its transformation product with zinc. Curve A: cortisol. Curve B: reaction product.

Table 4. *Some properties of model compounds treated with zinc*

Compound treated	Ratio of extinction of product to that of starting material (%)				
	At λ_{max} . (237–242 m μ .)	At 430 m μ . with Porter– Silber reagent	At 520 m μ . (corrected) with Zimmermann reagent		
			Direct	After BiO ₃ [–] treatment	After BH ₄ [–] /BiO ₃ [–] treatment
17 α :21-Dihydroxypregn-4-ene-3:11:20-trione (cortisone)	4	2	—	25	—
Cortisone acetate	4	2.5	—	—	—
11 β :17 α :21-Trihydroxypregn-4-ene-3:20-dione (cortisol)	6	61	—	19	—
Cortisol acetate	0.3	19	—	—	—
21-Acetoxy-17 α -hydroxypregn-4-ene-3:20-dione	2	2	—	—	—
21-Hydroxypregn-4-ene-3:20-dione	3	—	—	—	—
Pregn-4-ene-3:20-dione	0.6	—	—	—	—
11 α -Hydroxypregn-4-ene-3:20-dione	2.5	—	—	—	—
Pregn-4-ene-3:11-20-trione	4	—	—	—	—
Androst-4-ene-3:17-dione	4	—	105	—	—
Androst-4-ene-3:11:17-trione	2	—	—	—	—
11 β -Hydroxyandrost-4-ene-3:17-dione	0.3	—	—	—	—
3 β -Hydroxyandrost-5-en-17-one	—	—	110	—	—
3 α :17 α :20 β -Trihydroxypregnane-11-one	—	—	—	102	—
17 α :20 β :21-Trihydroxypregn-4-en-3-one	—	—	—	82	—
3 α :17 α -Dihydroxypregnane-11:20-dione	—	—	103	99	94
21-Acetoxy-3 α :17 α -dihydroxypregnane-11:20-dione	—	—	250	110	134

Addition of model compounds to the urine. Cortisone (10–50 $\mu\text{g./ml.}$), 3 α :17 α :20 β -trihydroxypregnan-11-one (18.2 $\mu\text{g./ml.}$), and 17 α :20 β :21-trihydroxypregn-4-en-3-one (19.8 $\mu\text{g./ml.}$) were added to urine samples, which were then assayed for 17-KGS and Zn/17-KGS. Treatment with Zn reduced the conversion of cortisone into a Zimmermann chromogen

from 78–88 to 7–10%, but failed to affect the 17-ketogenic property of the two other model compounds (see Table 6).

Thirty-four randomly chosen urine specimens were assayed for Zn/17-KGS. The results were in the range of 1.3–12.2 $\mu\text{g./ml.}$ (mean 5.3 $\mu\text{g./ml.}$). After the addition of cortisone (10 $\mu\text{g./ml.}$) to each specimen the average increment of Zn/17-KGS was $0.45 \pm 0.85 \mu\text{g./ml.}$ This increment was found to be statistically significant at the 99% probability level.

Treatment of pre-reduced urines with Zn. Each of three urine specimens was reduced with borohydride, then treated with Zn by the general procedure and finally oxidized with bismuthate. In parallel experiments the treatment with Zn was omitted. Each determination was carried out in quadruplicate. In Table 7 the results are given as mean values and their standard deviations.

Precision of routine assays. 17-KS, total 17-KS, Zn/total 17-KS, and 17-hydroxycorticosteroids (17-OH CS; cf. Appleby *et al.* 1955) were determined in duplicate in eighty-two randomly chosen urine specimens. Statistical evaluation of the results (see Table 8) shows that treatment with Zn does not seem to introduce a new systematic error but that the percentage error of the derived values for Zn/17-KGS and SDHA is considerable.

Table 5. Determination of 17-KS and total 17-KS in urine treated with zinc for various times

Reaction time (min.)	17-KS ($\mu\text{g./ml.}$)		Total 17-KS ($\mu\text{g./ml.}$)	
	Urine A	Urine B	Urine A	Urine B
0	20.6	8.4	35.8	14.5
30	20.6	9.2	30.3	13.2
60	20.0	8.6	27.5	11.8
90	—	8.5	—	11.5
120	20.6	—	27.7	—
180	19.7	—	27.4	—

Table 6. Contributions of model compounds to the determination of 17-KGS and Zn/17-KGS in the urine

Steroid added to the urine ($\mu\text{g./ml.}$)	Recovery	
	As 17-KGS (%)	As Zn/17-KGS (%)
Cortisone: 10	79	10
20	88	7
30	80	10
40	79	10
50	78	7
3 α :17 α :20 β -Trihydroxypregnan-11-one: 18.2	98	103
17 α :20 β :21-Trihydroxypregn-4-en-3-one: 19.8	92	99

Table 7. Treatment of pre-reduced urines with zinc

Urine	Zimmermann chromogens in terms of dehydroepiandrosterone after treatment with	
	$\text{BH}_4^-/\text{BiO}_3^-$ (mg./24 hr.)	$\text{BH}_4^-/\text{Zn}/\text{BiO}_3^-$ (mg./24 hr.)
C	15.1 ± 0.4	15.2 ± 1.0
D	41.8 ± 2.6	40.1 ± 2.2
E	16.7 ± 1.3	16.9 ± 0.6

DISCUSSION

The functional groups of urinary steroids comprise olefinic bonds, hydroxyl groups and keto groups. As far as could be ascertained (cf. Nes & Mason, 1951; Ercoli & Ruggieri, 1952; Fieser, 1953; Sondheimer, Kaufman, Romo, Martinez & Rosenkranz, 1953; Amandolla, Rosenkranz & Sondheimer, 1954; Barton & Robinson, 1954; Rosenfeld & Gallagher, 1955; Norzymberski, 1956; this paper) any one of these functions in isolation resists the action of zinc in glacial or aqueous acetic acid even under the most vigorous conditions (prolonged heating at reflux and large excess of the reagent). However, when any two of these functions are placed at adjacent carbon atoms, the resulting groupings (α -ketols, allylic alcohols, α : β -unsaturated ketones) can be brought to react with zinc under appropriate conditions. Though an exception has been found in the behaviour of 17:20-ketols and other exceptions are possible, the generalization is useful in indicating which urinary steroids are

Table 8. Statistical evaluation of eighty-two duplicate assays

	Mean ($\mu\text{g./ml.}$)	Range ($\mu\text{g./ml.}$)	Standard deviation (s)* ($\mu\text{g./ml.}$)	Coeff. of variation (%)
Total 17-KS (a)	16.3	7.2–52.0	0.85	5
Zn/total 17-KS (b)	12.3	5.9–43.7	0.765	6
17-KS (c)	6.7	2.8–22.5	0.195	3
17-OH CS (d)	10.0	3.8–38.6	0.57	6
17-KGS (e)	9.6	3.0–42.0	0.86	9
Zn/17-KGS (f)	5.6	1.4–21.2	0.78	14
SDHA (g)	4.0	0–28.0	1.13	28

* s_a , s_b , s_c and s_d were calculated as $\frac{\text{average within sample range}}{1.13}$; $s_e = \sqrt{(s_a^2 + s_c^2)}$; $s_f = \sqrt{(s_b^2 + s_c^2)}$; $s_g = \sqrt{(s_a^2 + s_b^2)}$.

likely to undergo transformations when the urine is treated with zinc, and how such transformations will affect the proposed differentiation of urinary 17-ketogenic steroids. For instance, if it is assumed that urinary α -ketols other than 17:20-ketols are converted by zinc into the corresponding ketones and by bismuthate into carboxylic acids, then consecutive treatment with zinc and bismuthate will give rise to an increase of ketonic components in the neutral fraction of the reaction products and hence to an increase of Zimmermann chromogens measured in the assay of Zn/17-KGS. The latter increase will be relatively large if the products obtained from α -ketols are 17-oxosteroids.

For reasons stated above, and because dihydroxyacetones on treatment with zinc and then with bismuthate retain some chromogenic property in the Zimmermann reaction, the assay of Zn/17-KGS will tend to overestimate the urinary levels of 17:20-glycols and 17:20:21-triols, and consequently the values calculated for urinary dihydroxyacetones will underestimate the actual amount of this group of compounds. Further, in its present form, the proposed assay suffers from a lack of precision (see Table 8). Nevertheless the results so far obtained with its application to clinical material (see Fig. 3) clearly demonstrate its usefulness.

SUMMARY

1. Zinc powder in boiling aqueous acetic acid was found to effect the reductive removal of (i) the keto group in 4:5-unsaturated 3-oxosteroids, (ii) the 17- and 21-hydroxyl groups in 17 α :21-dihydroxy-20-oxosteroids, and (iii) the 21-acetoxyl group in 21-acetoxy-17 α -hydroxy-20-oxosteroids. Among the types of compounds resistant to the action of zinc were 17-ketones, 17:20-diols and 17:20:21-triols.

2. An analytical method based on these findings was developed for the differential estimation of zinc-resistant (17:20-diols and 17:20:21-triols) and zinc-labile (dihydroxyacetones) 17-ketogenic steroids.

3. Urine specimens obtained from men and

women in normal health, women in the last trimester of pregnancy, rheumatoid arthritics receiving cortisone acetate or corticotrophin and patients with adrenogenital syndrome were assayed by the analytical method described.

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