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Indirect Analysis of Corticosteroids

1. THE DETERMINATION OF 17-HYDROXYCORTICOSTEROIDS

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For the present purpose it is convenient to classify the known 17-hydroxycorticosteroids, according to the mode of substitution at the triad $C_{(17)}$–$C_{(20)}$–$C_{(21)}$, into the following four structural types: (*A*) 21-deoxy-17:20-ketols,* (*B*) 21-hydroxy-17:20-ketols (dihydroxyacetones), (*C*) 21-deoxy-17:20-glycols, and (*D*) 21-hydroxy-17:20-glycols (glycerols) (see Table 1). The last two groups (*C* and *D*) are degraded by periodate to 17-oxosteroids (cf. Reichstein & Shoppee, 1943); their indirect determination by measurement of the formed 17-oxosteroids as chromogens with *m*-dinitrobenzene and alkali (Zimmermann, 1935, 1936) was described by Talbot & Eitingon (1944) and by Fieser, Fields & Lieberman (1944). More recently Brooks & Norymberski (1952, 1953) have shown that bismuthate not only effects the above oxidations but also converts dihydroxyacetones (*B*) into 17-ketones and, on this basis, have developed an analytical procedure for the determination of 17-ketogenic steroids (types *B*, *C* and *D*).

The present report concerns a further development of the indirect analysis of 17-hydroxycorticosteroids by their conversion into 17-oxosteroids. The principal feature of this development consists in submitting an analytical sample to a sequence of reactions, the last of which is the oxidative fission of glycols, the presence and derivation of the latter being determined by the preceding operations. In particular, the following two analytical schemes have been evolved.

* Strictly applied the prefix 'deoxy' signifies the removal of an oxygen function already denoted in the name to which the prefix is added. Here, the term is used to indicate the absence of oxygen from a position at which it often occurs in closely related compounds; this provides a simple and unambiguous description for groups of compounds which will be frequently mentioned in the text.

Determination of all 17-hydroxycorticosteroids (17-OHCS)

The analytical sample is reduced under conditions ensuring the quantitative conversion of ketones into alcohols, and is subsequently treated with sodium bismuthate: in the reductive step the 17:20-ketols (*A* and *B*) are converted into the corresponding 17:20-glycols (*C* and *D*), the latter, whether thus formed or originally present, are degraded to 17-ketones in the oxidative step. The total effect of this reaction sequence is the conversion of all 17-hydroxycorticosteroids (*A*, *B*, *C* and *D*) into 17-oxosteroids, whilst all other ketones originally present, including 17-oxosteroids, are reduced to alcohols. (Since the term '17-hydroxycorticosteroids' is currently employed to denote chromogens in the reaction of Porter & Silber (1950), i.e. dihydroxyacetone derivatives (*B*), it is suggested that, whenever confusion of terms is likely to occur, the group of compounds determined by the present method should be referred to as 'total 17-hydroxycorticosteroids'.)

Selective determination of 21-*deoxy*-17:20-*ketols*

The analytical sample is treated with sodium bismuthate whereby 21-deoxy-17:20-ketols (*A*) remain unchanged while all other 17-hydroxycorticosteroids (*B*, *C* and *D*) are converted into 17-ketones. Subsequently the mixture is submitted to the reaction sequence described in the foregoing method, with the result that all 17-oxosteroids present in the final mixture of products are derived exclusively from 21-deoxy-17:20-ketols (*A*).

Each of the proposed schemes involves, at some stage, the reduction of ketones to alcohols. Sodium borohydride, which was first introduced by Chaikin & Brown (1949) for the selective reduction of

ketones, aldehydes and acid chlorides under mild conditions, seemed most suitable for our purpose. The reagent acts in aqueous or non-aqueous media, is simple to use, and does not cause side reactions even when present in large excess. Moreover, sodium borohydride has been extensively and successfully used for the reduction of oxosteroids (for references see Brown & Mead, 1953) including the reduction of 21-deoxy-17:20-ketols to 21-deoxy-17:20-glycols (Herzog, Jevnik, Perlman, Nobile & Hershberg, 1953; Oliveto & Hershberg, 1953) and of dihydroxyacetones to 17:20:21-triols (Norymberski & Woods, 1954).

isolating the tetrol III, the reduction mixture was treated with sodium bismuthate in the presence of acetic acid 3α:11β-dihydroxy-5β-androstan-17-one (V) was obtained in good yield. The same sequence of reactions—reduction with borohydride followed by oxidation with bismuthate—likewise smoothly converted 21-acetoxy-3α:17α-dihydroxypregnane-11:20-dione (I*b*) into 3α:11β-dihydroxy-5β-androstan-17-one (V), while in the direct treatment of I*b* with bismuthate only the starting material was recovered. The last finding is of incidental interest, since it supports the view (Brooks & Norymberski, 1953) that the degradation of dihydroxyacetones

Table 1. *Summary of methods for the determination of* 17-*hydroxycorticosteroids by their conversion into* 17-*oxosteroids*

Treated with	Structural type						Designation of determined group
	21 CH_3	CH_2OH	CH_3	CH_2OH			
	20 CO	CO	CHOH	CHOH	O	Other ketones *	17-Ketosteroids (17-KS)
(Untreated)	17 C..OH	C..OH	C..OH	C..OH	C		
	*A**	*B*	*C*	*D*	**		
	CH_3						
	CO	COOH	O	O	O	Other ketones *	17:20-Glycols†
IO_4^-	C..OH	C..OH	C	C	C		
	*		**	**	**		
	CH_3						
	CO	O	O	O	O	Other ketones *	17-Ketogenic steroids (17-KGS†)
BiO_3^-	C..OH	C	C	C	C		
	*	**	**	**	**		
	O	O	O	O	OH	Other alcohols	
1. BH_4^-	‖	‖	‖	‖	|		17-Hydroxycorticosteroids (total 17-OH CS)
2. BiO_3^-	C	C	C	C	CH		
	**	**	**	**			
	O	OH	OH	OH	OH	Other alcohols	
1. BiO_3^- 2. BH_4^- 3. BiO_3^-	‖	|	|	|	|		21-Deoxy-17:20-ketols
	C	CH	CH	CH	CH		
	**						

† By subtraction of 17-KS.
** Major contribution to the Zimmermann colour (17-oxosteroids)
* Minor contribution to the Zimmermann colour (other ketones).

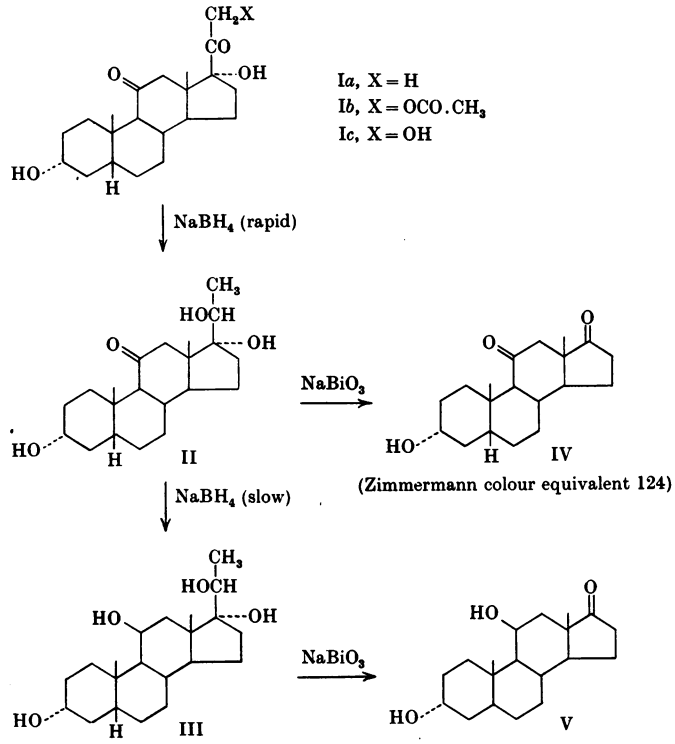
We have now carried out a few preparative reductions with borohydride under conditions suitable for adaptation to an analytical procedure. Treatment of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) with sodium borohydride in aqueous *tert.*-butanol at room temperature afforded pregnane-3α:11β:17α:20β-tetrol (III) in high yield, in agreement with the findings of Herzog *et al.* (1953), who used aqueous methanol as solvent. When, without isolation, the tetrol III was treated with sodium bismuthate in the presence of acetic acid, 3α:11β-dihydroxy-5β-androstan-17-one (V) was obtained in good yield. The same sequence of reactions—reduction with borohydride followed by oxidation with bismuthate—likewise smoothly converted 21-acetoxy-3α:17α-dihydroxypregnane-11:20-dione (I*b*) into 3α:11β-dihydroxy-5β-androstan-17-one (V), while in the direct treatment of I*b* with bismuthate only the starting material was recovered. The last finding is of incidental interest, since it supports the view (Brooks & Norymberski, 1953) that the degradation of dihydroxyacetones (*B*) to 17-ketones by bismuthate depends upon the initial fission of the $C_{(20)}$–$C_{(21)}$ bond and formation of an α-hydroxy acid.

The analytical experiments were performed under conditions closely following those employed for the preparative reactions. The progress of the reduction of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) was determined by measuring, at intervals, the amount of 20-ketones in the reduction mixture, and

the amount of 17-ketones after oxidation with bismuthate, both measurements being performed by means of the Zimmermann reaction. It was found that the amount of Zimmermann chromogens in the reduction mixture decreased to negligible values within 5 min. or less (Fig. 1, curve 1), while the chromogens formed on subsequent oxidation with bismuthate reached a maximum value within *ca.* 10 min. and then gradually fell to a constant level attained after a reduction time of *ca.* 7 hr. (curve 2).

treatment with a large excess of sodium borohydride. The method was found to account satisfactorily for varying amounts (35–180 μg.) of a model compound (ketol I*a*) and to be unaffected by the presence of a ketone (dehydro*epi*androsterone).

Our attention was next directed towards the direct reduction of oxosteroids in urine: it was found that, under appropriate conditions, the reaction is readily brought about; it provides, in conjunction with the method previously developed



These observations are consistent with the rapid reduction of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) to 3α:17α:20β-trihydroxypregnan-11-one (II) and the latter's slow reduction to pregnane-3α:11β:17α:20β-tetrol (III); treatment with bismuthate converts the initial product (II) into 3α-hydroxy-5β-androstane-11:17-dione (IV) and the final product (III) into 3α:11β-dihydroxy-5β-androstan-17-one (V). The Zimmermann colour equivalents of the oxidation products IV and V are respectively 124 (Brooks & Norymberski, 1953) and 85 (see p. 457) (dehydro*epi*androsterone ≡ 100), and hence the shape of curve 2.

A standard analytical procedure for the determination of 17-hydroxycorticosteroids (17-OH CS) was next developed wherein the complete reduction of all ketones is effected by prolonged (overnight)

for the oxidation of ketogenic steroids in urine (Norymberski, 1952; Norymberski, Stubbs & West, 1953), a simple means of assaying urinary 17-OH CS. The adopted standard method comprises the overnight treatment of urine with a large excess of sodium borohydride (5 mg./ml.), followed by oxidation with bismuthate, the formed 17-oxosteroids being taken as a direct measure of urinary 17-OH CS. The following observations bear on the method's efficacy: (i) Native 17-oxosteroids as well as comparatively very large amounts of those added to the urine (dehydro*epi*androsterone, 44·5 μg./ml.) were quantitatively reduced in the assay. (ii) Addition of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) and of 3α:17α:21-trihydroxypregnane-11:20-dione (I*c*) resulted in an increase of Zimmermann chromogens accounting for 83% (mean value) of

the added compounds; reduction of the 20-oxo group in I*a* being prerequisite for its subsequent conversion into a 17-oxosteroid, it is concluded that under the conditions of the *in situ* treatment 20-ketones are reduced as well as the 17-ketones. (iii) Addition of dehydro*epi*androsterone (50 μg./ml.) to several randomly chosen urines (pH range 5·1–7·8) in no case produced any significant increase of the determined 17-OH CS.

(ii) If, as can be reasonably expected, 11-ketones are reduced by borohydride under the conditions used, then the Zimmermann chromogens measured in the two assays comprise two different ranges of individual 17-oxosteroids with varying chromogenic contributions. For this reason alone an accurate correlation of the two assays is impossible. An approximation may be attempted by assuming that 3α:17α:21-trihydroxypregnane-11:20-dione (I*c*)

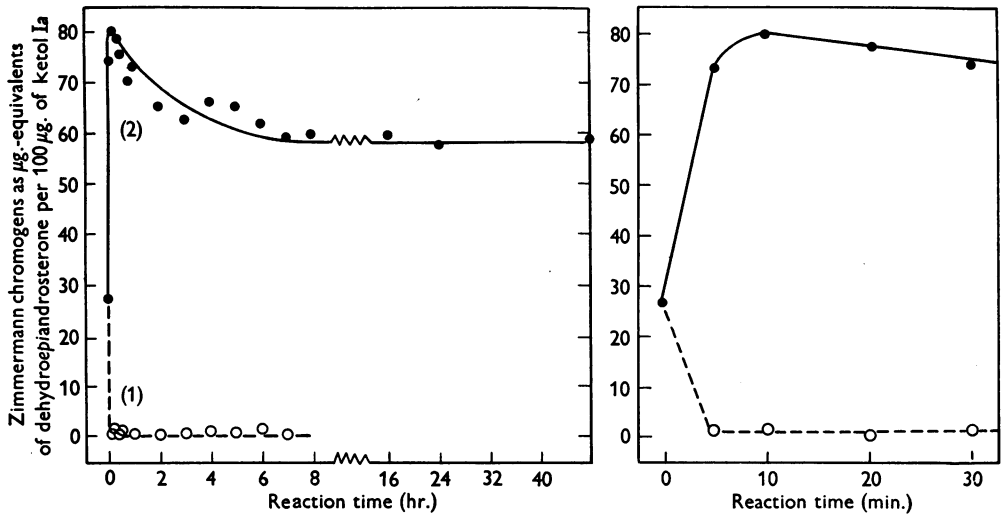


Fig. 1. The rate of reduction of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) with sodium borohydride. Curve (1): Zimmermann chromogens in the reduced mixture; curve (2): Zimmermann chromogens in the reduced and subsequently oxidized mixture. The right-hand figure shows the changes over the period 0–30 min. on a larger scale.

Assays of 17-OH CS were performed, concurrently with those of 17-ketogenic steroid (17-KGS), on 88 urines, 27 of which were obtained from normal subjects and 61 from rheumatoid patients treated with cortisone, cortisol, or adrenocorticotrophic hormone. In the majority of cases the results of the two assays, expressed in terms of dehydro*epi*androsterone, agreed within the limits of the experimental error of either assay. The mean daily excretion of 17-KGS for the whole group was 22·4 mg. (range 5·8–59·2 mg.), that of 17-OH CS was 23·1 mg. (range 8·1–62·8 mg.). It must be emphasized here that agreement or disagreement between the results of the two assays cannot be interpreted without carefully considering all factors contributing to each group of results; of these the following occur to us: (i) The contributions of 21-deoxy-17:20-ketols to the results of 17-OH CS assays are normally so small that they may be ignored in the present consideration. However, in a case of adrenogenital syndrome the abnormally high excretion of 21-deoxy-17:20-ketols was reflected in a considerable preponderance of 17-OH CS over 17-KGS (cf. Appleby & Norymberski, 1955). is the main urinary 17-hydroxycorticosteroid. This compound is measured as 3α-hydroxy-5β-androstane-11:17-dione (IV) in the 17-KGS assay and as 3α:11β-dihydroxy-5β-androstan-17-one (V) in the 17-OH CS assay. The colour equivalents of the two 17-ketones being 124 and 85 respectively it would follow that the measure of I*c* as 17-KGS exceeds that as 17-OH CS by 45%. In fact this calculation is not entirely correct since, during the hot acid hydrolysis employed in the assay, 3α:11β-dihydroxy-5β-androstan-17-one (V) is presumably partly dehydrated to 3α-hydroxy-5β-androst-9-(11)-en-17-one, a 17-ketone with the colorimetric equivalent of 117 (Wilson, 1950). (iii) It was shown that 21-acetoxy-3α:17α-dihydroxypregnane-11:20-dione (I*b*) is resistant to treatment with bismuthate, but that it is smoothly converted into 3α:11β-dihydroxy-5β-androstan-17-one (V) if the oxidation is preceded by reduction with borohydride: the compound could therefore be determined as a 17-OH CS but not as a 17-KGS. Most likely dihydroxyacetones with the 21-hydroxyl masked by, e.g. conjugation with sulphuric acid or with glucuronic acid will exhibit the same property. Such

compounds, if present in the urine, would contribute only to the 17-OH CS values. (iv) Urinary pigments contribute to a varying extent to the colorimetric measurement of native 17-oxosteroids (17-KS); they are, however, transformed by bismuthate into colourless products and consequently do not interfere with the assay of 17-oxosteroids in bismuthate-treated urines. Since 17-KGS values are computed by subtracting 17-KS from total 17-oxosteroids, they are, in contradistinction to 17-OH CS, affected by pigments. It is concluded that the observed agreement between the excretion of 17-KGS and 17-OH CS results from the interplay of opposing factors, and that disagreement between the results of the two assays does not necessarily reflect on the reliability of either method nor indicate the presence of urinary steroids which can be assayed by the one and not by the other method. For routine purposes the two methods may be equated in most cases since, in the absence of abnormally large quantities of 21-deoxy-17:20-ketols, they both provide a measure of the same group of urinary corticosteroids. The determination of 17-OH CS has however the advantage of increased accuracy since the result is obtained from a single colorimetric measurement instead of being computed from two such measurements. Moreover, this determination is more rapidly performed, one person being able to assay (in duplicate) twelve urines in one day.

The present work was briefly reported in a preliminary communication (Appleby, Gibson, Norymberski & Stubbs, 1954). The proposed selective determination of 21-deoxy-17:20-ketols is reported in the following communication (Appleby & Norymberski, 1955).

EXPERIMENTAL

Melting points were determined on a Kofler stage. Microanalyses are by Weiler and Strauss, Oxford; samples for analyses were dried overnight at 100° in a high vacuum. Specific rotations were measured at 15–20°. Determinations of Zimmermann chromogens were performed by the procedure of Zygmuntowicz, Wood, Christo & Talbot (1951): a colour correction (Talbot, Berman & McLachlan, 1942) was applied. Sodium bismuthate was of AnalaR grade (British Drug Houses, Ltd.).

Preparations

Pregnane-3α:11β:17α:20β-tetrol (III)

A solution of 3α:17α-dihydroxypregnane-11:20-dione (I*a*) (203 mg.) in aqueous *tert.*-butanol (80%, v/v; 25 ml.) was treated with $NaBH_4$ (200 mg.) for 5·5 hr. at room temperature. Aqueous acetic acid (5%, v/v; 10 ml.) and water (50 ml.) were added and the mixture was extracted with $CHCl_3$ (1 × 50 ml., 3 × 25 ml.). The combined extract was washed successively with 2N-Na_2CO_3 (25 ml.) and water (2 × 25 ml.), each washing being back-extracted with $CHCl_3$ (20 ml.). Crystallization of the crude product from methanol–acetone gave small prisms (118 mg.), m.p. 279–281°; concentration of the mother liquors afforded 32 mg. of less pure material, m.p. 274–280°. Further crystallization from methanol raised the m.p. to 282–284°, $[\alpha]_D$ +23·5° in dioxan (*c*, 0·64). (Found: C, 71·3; H, 10·4. Calc. for $C_{21}H_{36}O_4$: C, 71·6; H, 10·3%.) The reported m.p. of III is 275–282° (Herzog *et al.* 1953.)

3α:11β-Dihydroxy-5β-androstan-17-one (V)

From 3α:17α-*dihydroxypregnane*-11:20-*dione* (I*a*). A solution of I*a* (105 mg.) in aqueous *tert.*-butanol (80%, v/v; 5 ml.) was treated with $NaBH_4$ (100 mg.) overnight at room temperature. Aqueous acetic acid (50%, v/v; 100 ml.) and $NaBiO_3$ (5 g.) were added and the mixture was shaken for 1 hr. The surplus reagent was reduced with a 10% $Na_2S_2O_5$ solution (20 ml.) and, after addition of 3N-NaOH (100 ml.), the product was extracted with ether (2 × 200 ml.), the extract washed successively with water (50 ml.), 3N-NaOH (100 ml.), and water (2 × 50 ml.), dried over Na_2SO_4, and brought to dryness. The crystalline residue (90 mg.; m.p. 215–225°) furnished, after two crystallizations from acetone–light petroleum, pure 3α:11β-dihydroxy-5β-androstan-17-one (57 mg.), m.p. 235–237°; chromatography of the mother liquors on Al_2O_3 gave 10 mg. more of identical material. $[\alpha]_D$ +100° in dioxan (*c*, 1·00), $[\alpha]_D$ +105° in $CHCl_3$–ethanol (4:1, by vol.) (*c*, 1·17). (Found: C, 74·4; H, 9·8. Calc. for $C_{19}H_{30}O_3$: C, 74·5; H, 9·9%.)

From 21-*acetoxy*-3α:17α-*dihydroxypregnane*-11:20-*dione* (I*b*). Treatment of I*b* (120 mg.) under conditions employed in the foregoing preparation afforded pure 3α:11β-dihydroxy-5β-androstan-17-one (81 mg.), m.p. 235–236°, $[\alpha]_D$ +108° in $CHCl_3$–ethanol (4:1, v/v) (*c*, 0·87). (Found: C, 74·3; H, 9·9. Calc. for $C_{19}H_{30}O_3$: C, 74·5; H, 9·9%.) Herzog *et al.* (1953) recorded for this compound m.p. 237–238·6° and $[\alpha]_D$ +104° in dioxan.

The colour extinction of V in the Zimmermann reaction was found equivalent to 0·85 mol. prop. of dehydro*epi*-androsterone.

Treatment of 21-*acetoxy*-3α:17α-*dihydroxypregnane*-11:20-*dione* (I*b*) *with sodium bismuthate*

A solution of I*b* (95 mg.) in aqueous acetic acid (50%, v/v; 20 ml.) was shaken for 1 hr. with $NaBiO_3$ (1 g.). The mixture was worked up as specified above using $CHCl_3$ (5 × 20 ml.) for the extraction. The crude product gave from ethyl acetate crystals (75 mg.), m.p. 227–230°, undepressed on admixture with starting material (I*b*) of the same m.p., $[\alpha]_D$ +78° in acetone (*c*, 1·00). The Zimmermann test failed to detect any 3α-hydroxy-5β-androstane-11:17-dione (IV) in the mother liquors.

Determination of 17-*hydroxycorticosteroids*

General procedure

The analytical sample is placed in a glass-stoppered centrifuge tube (approx. capacity 30 ml.) and dissolved in aqueous *tert.*-butanol (80%, v/v; 0·2 ml.). Directly before use a solution of $NaBH_4$ is prepared by dissolving the reagent (100 mg.) in water (1 ml.) and adjusting the volume to 5·0 ml. with *tert.*-butanol. This solution (0·3 ml.) is added to the reaction tube and the mixture left overnight at room temperature. Aqueous acetic acid (50%, v/v; 2 ml.) and $NaBiO_3$ (0·2 g.) are added, the tube is shaken for 0·5 hr. and,

after addition of a 6% $Na_2S_2O_5$ solution (2·5 ml.), the shaking is continued until all $NaBiO_3$ has been reduced. 10N-HCl (0·5 ml.) is added and the mixture extracted by shaking for 15 min. with ethylene dichloride (10·0 ml.). The phases are separated by centrifuging and the top layer is removed with suction. The extract is shaken first with water (2·5 ml.) for 2 min. and then with 3N-NaOH (2·5 ml.) for 5 min., after each wash the phases are separated as described above. The washed extract is filtered through a fluted paper: from the filtrate two samples of 4·0 ml. are taken for the measurement of Zimmermann chromogens. At suitable concentrations (10–40 μg.-equiv. of dehydro*epi*androsterone for Zimmermann reaction) the results of duplicate assays differed by not more than ±6%.

Variation of the reduction time

Determination of unreacted 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *as Zimmermann chromogen.* Eleven samples of the ketol I*a* (448 μg. each) were treated with $NaBH_4$ as in the general procedure. The reduction of each sample was stopped, by addition of aqueous acetic acid (5%, v/v; 5 ml.), after reaction times of 5 min. to 7 hr. Extraction with ethylene dichloride and all following operations were performed as in the general procedure. A further sample of I*a* (448 μg.) was processed in the same manner but omitting the addition of $NaBH_4$. The eleven reduced samples gave readings equivalent to 0·4–2·6 μg. dehydro*epi*androsterone. The results indicate clearly the complete or almost complete reduction of the 20-oxo group (see Fig. 1, curve 1, p. 456), although at this concentration the colorimetric measurements are grossly inaccurate (mean variation between duplicates *ca.* 100%).

Determination of reacted 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *as* 17-*ketogenic steroid.* Sixteen samples of the ketol I*a* (89·5 μg. each) were treated with $NaBH_4$ as in the general procedure. The reduction of each sample was stopped, by addition of aqueous acetic acid (50%, v/v; 2 ml.), after reaction times of 5 min. to 48 hr. All subsequent operations followed the general procedure. The results recorded in Fig. 1 (curve 2) are means of duplicate determinations.

Determination of 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *at varying concentrations and in the presence of dehydro*epi*androsterone* (*DHA*)

All determinations were carried out by the general procedure. The results recorded in Table 2 are means of duplicate determinations. The recoveries were calculated on the basis of a quantitative conversion of the ketol (I*a*) into 3α:11β-dihydroxy-5β-androstan-17-one (V) and the latter's colour equivalent of 85. The approximately linear relationship between measurements at different concentrations of I*a* is shown in Fig. 2.

Table 2. *Determination of* 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *at varying concentrations and in the presence of dehydro*epi*androsterone* (*DHA*)

I*a* (μg./sample)	DHA/sample (μg.)	DHA μg.-equiv. found	Recovery (%)
35·7	—	22·7	89
53·7	—	36·7	97
71·5	—	43·2	86
89·5	—	53·7	85
134·2	—	77·2	82
179·0	—	103·7	82
89·5	50	53·8	85
89·5	94	58·0	92
89·5	188	56·8	90
89·5	282	57·0	90
89·5	470	57·5	91

Determination of 17-*hydroxycorticosteroids in the urine*

General procedure. The volume of a 24 hr. urine specimen (collected and stored without addition of a preservative) is adjusted to 2·0 l. with distilled water. A sample (4·0 ml.) of the diluted urine is placed in a glass-stoppered centrifuge tube (approx. capacity 50 ml.), $NaBH_4$ (20 mg.) is added and the solution left to stand overnight at room temperature. Glacial acetic acid (4 ml.) and $NaBiO_3$ (1 g.) are added, the tube is shaken for 0·5 hr. and, after addition of a 6% $Na_2S_2O_5$ solution (10 ml.), the shaking is continued until all $NaBiO_3$ has been reduced. 10N-HCl (6 ml.) is added, the tube placed in a boiling-water bath for 10 min. and then cooled in cold water. The extraction (with 20·0 ml. ethylene dichloride) and washing of the extract (with 5 ml. water and then with 5 ml. 3N-NaOH) are performed as described above. The determination is carried out on duplicate samples. For routine purposes twelve urine specimens are assayed concurrently. At suitable concentrations (10–40 μg.-equiv. of dehydro*epi*androsterone for Zimmermann reaction) the results of duplicate assays differed by not more than ±6%.

Variation of quantity of sodium borohydride. To each of ten samples (4·0 ml.) of a urine specimen aqueous *tert.*-butanol (80%, v/v; 0·4 ml.) was added. Varying amounts (1–20 mg.) of $NaBH_4$ were added to nine samples; after standing overnight at room temperature all ten samples were processed by the general procedure. In a second series of experiments a solution of dehydro*epi*androsterone (178 μg.) in aqueous *tert.*-butanol (80%, v/v; 0·4 ml.) was added to each urine sample instead of the solvent alone, while all other experimental conditions remained unchanged. Replicate (3–6) determinations were carried out. It was found that when more than 8 mg. $NaBH_4$ (2 mg./ml.

Table 3. *Determinations of* 17-*hydroxycorticosteroids* (17-*OH CS*) *in urines with and without addition of dehydro*epi*androsterone* (*DHA*)

All values are in terms of equivalents of dehydro*epi*androsterone formed. pH values of urines 5·1–7·8.

Found 17-OH CS (μg./ml.)	
Urine alone	Urine + DHA*
12·6	13·2
10·5	11·3
9·3	9·5
7·9	8·9
14·0	15·9
5·3	5·6
27·2	27·2
27·0	27·3
6·5	7·4
8·0	9·2
6·5	5·8

* 50 μg./ml. DHA added.

urine) were used, the results of both experimental series were practically identical.

Determination of urinary 17-hydroxycorticosteroids in the presence of added dehydroepiandrosterone. All urine specimens were assayed by the general procedure. Dehydroepiandrosterone (200 μg.) in aqueous *tert.*-butanol (80%, v/v; 0·4 ml.) or the solvent alone were added to the investigated samples. The results are recorded in Table 3.

Table 4. *Recoveries of* 3α:17α-*dihydroxypregnane*-11:20-*dione* (*Ia*) *and of* 3α:17α:21-*trihydroxypregnane*-11:20-*dione* (*Ic*) *from urine*

Steroid (μg./sample)	Recovery (%)
9·9 (Ia)	66
19·8 (Ia)	82
39·6 (Ia)	85
59·4 (Ia)	86
79·2 (Ia)	83
9·7 (Ic)	75
19·4 (Ic)	84
38·8 (Ic)	80
58·2 (Ic)	84
77·6 (Ic)	77

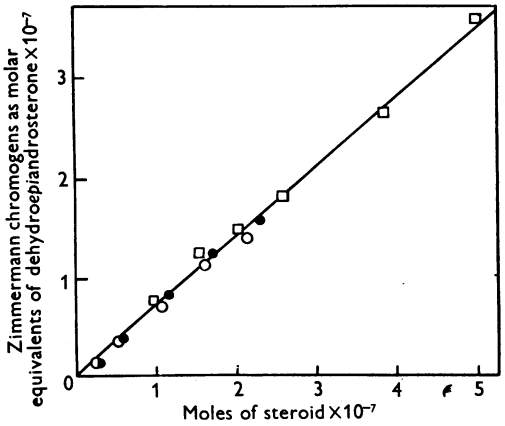


Fig. 2. Proportionality in the determination of 17-OH CS by their conversion into 17-oxosteroids. (□): 3α:17α-dihydroxypregnane-11:20-dione (*Ia*) in *tert.*-butanol; (●): *Ia* in the urine; (○): 3α:17α:21-trihydroxypregnane-11:20-dione (*Ic*) in the urine.

Recovery of 17-hydroxycorticosteroids added to the urine. Varying amounts of 3α:17α-dihydroxypregnane-11:20-dione (*Ia*) and of 3α:17α:21-trihydroxypregnane-11:20-dione (*Ic*) respectively were dissolved in 0·4 ml. portions of aqueous *tert.*-butanol (80%, v/v) and the solutions added to samples of a urine specimen. The determinations were performed by the general procedure. The results are recorded in Table 4 and in Fig. 2. Recoveries were calculated on the basis of quantitative conversions of both model compounds into 3α:11β-dihydroxy-5β-androstan-17-one (V) and the latter's colour equivalent of 85. In the present case this calculation can be only approximate since, during the hot acid hydrolysis, 3α:11β-dihydroxy-5β-androstan-17-one (V) is presumably partly dehydrated to 3α-hydroxy-5β-androst-9(11)-en-17-one, a 17-oxosteroid with a colour equivalent of 117 (Wilson, 1950).

DISCUSSION

Methods for the determination of 17-hydroxycorticosteroids by their conversion into 17-oxosteroids (see Table 1) have the merit of specificity since (i) the conversion is brought about by agents of specific and predictable action, and (ii) the formed 17-ketones are selectively measured as chromogens in the Zimmermann reaction. The latter statement must be qualified by considering the limitations of the Zimmermann reaction.

The formation of dyes with *m*-dinitrobenzene and alkali is a fairly general property of compounds containing activated methylene groups, including methylene (or methyl) ketones. However, if, as is customary, the reaction is performed at 25° for 1 hr. in absolute ethanol (Callow, Callow & Emmens, 1938; Medical Research Council, 1951) or in aqueous ethanol (Holtorff & Koch, 1940) high extinction at 520 mμ. is characteristic of 17-oxosteroids. Similar chromogenic properties were reported for cholestan-2-one (Broadbent & Klyne, 1954) and for 2:3:6-trimethylbenzalacetone (Holtz, 1954) but other steroid and non-steroid ketones so far examined show comparatively little absorption at 520 mμ. (Kaziro & Shimada, 1937; Callow *et al.* 1938; Langstroth & Talbot, 1939; Hansen, Cantarow, Rakoff & Paschkis, 1943; Broadbent & Klyne, 1954). In the determination of 17-hydroxycorticosteroids by the present method such atypical Zimmermann chromogens can derive only from compounds of structure $\rangle C(OH).C(OH)R^1R^2$ or $\rangle C(OH).CO.R^3$ wherein the site of attachment of the tertiary hydroxyl is not $C_{(17)}$ of a steroid nucleus.

A further limitation of the Zimmermann reaction arises from the variation of colour intensity among individual 17-oxosteroids; the observed variation is, relative to dehydroepiandrosterone (≡100), about 65 to 130 (Wilson, 1950, 1954; Brooks & Norymberski, 1953), but St André, MacPhillamy, Nelson, Shabica & Scholz (1952) reported the case of a 17-ketone (3β-acetoxy-14ζ-hydroxyandrost-5-en-17-one) which failed to produce any colour in the test. The non-equivalence of colour does not affect the specificity of analytical methods employing the Zimmermann reaction but it severely limits their quantitative evaluation.

It is evident that the selective conversion of 17-hydroxycorticosteroids into 17-ketones may be employed not only for the group determination of the former compounds but also for their more detailed analysis. Edwards, Kellie & Wade (1953) have fractionated on alumina 17-oxosteroids isolated from urine treated with sodium bismuthate

and by comparing the chromatographic patterns so obtained with those of native 17-oxosteroids were able to show that practically all 17-ketogenic steroids of the investigated urine were oxygenated at $C_{(11)}$. With the introduction of the *in situ* reduction of urinary ketones a simple differential analysis of urinary 17-hydroxycorticosteroids becomes feasible, wherein 17-oxosteroids formed from 17-hydroxycorticosteroids on successive treatment with borohydride and bismuthate are separated from all other urinary constituents and their transformation products and fractionated by well-established methods.

SUMMARY

1. A combination of reductive and oxidative operations was proposed as a means of selectively converting 17-hydroxycorticosteroids into 17-oxosteroids. The suitability of sodium borohydride for effecting the reductive step was demonstrated on the preparative and on the analytical scale.

2. The reaction sequence—reduction with borohydride, followed by glycol fission with bismuthate—was employed to develop a method for the determination of urinary total 17-hydroxycorticosteroids by their conversion into 17-oxosteroids in urine and measurement of the latter as chromogens in the Zimmermann reaction.

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Indirect Analysis of Corticosteroids

2. THE DETERMINATION AND IDENTIFICATION OF URINARY 17-HYDROXY-20-OXOSTEROIDS UNSUBSTITUTED AT $C_{(21)}$

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In the preceding paper (Appleby, Gibson, Norymberski & Stubbs, 1955) an analytical procedure was proposed for the determination of 17-hydroxy-20-oxosteroids unsubstituted at $C_{(21)}$ (21-deoxy-17:20-ketols = 21-deoxyketols) by their selective conversion into 17-oxosteroids and measurement of the latter as chromogens in the Zimmermann reaction. The proposed method comprises three reaction steps (see Fig. 1): (1) treatment with sodium bismuthate to convert all 17-hydroxycorticosteroids other than 21-deoxyketols into 17-ketones; (2) treatment with sodium borohydride to reduce all ketones, including those formed in the preceding step, to alcohols and, in particular, to