SUMMARY

1. A simple chromatographic method is presented for the separation of glutamic from aspartic acid, and their estimation, after their separation together from a protein hydrolysate, employing the synthetic anion exchange resin, Amberlite IR 4.

2. Optically active cysteic acid was isolated from a hydrolysate of chlorinated wool.

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The Polarographic Estimation of Steroid Hormones

4. DETERMINATION OF 3(α)- AND 3(β)-HYDROXY-17-KETOSTEROIDS

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A knowledge of the urinary excretion of 3(β)-hydroxy-17-ketosteroids (' β -17-ketosteroids') is becoming increasingly important in clinical diagnosis. In addition to the now well-established fact, first suggested by Crooke & Callow (1939) and by Talbot, Butler & Berman (1942), that the relative excretion of β -17-ketosteroids is markedly elevated in patients with adrenal cortical carcinoma, the work of Scott & Vermeulen (1942), Pearlman (1942), Dobriner, Gordon, Rhoads, Lieberman & Fieser (1942), Freeman, Pincus & Glover (1944), Salter, Cahen & Sappington (1946) and Dingemanse, Huis in't Veld & de Laat (1946) has shown that the excretion of β -17-ketosteroids may be altered in various other cancerous and non-cancerous conditions. It therefore appeared desirable to extend the polarographic method for the estimation of 17-ketosteroids (Barnett, Henly & Morris, 1946) so that it could also be used for the routine estimation of the α - and β -17-ketosteroids.

Several methods have been described for the quantitative separation of the 3(α)- and 3(β)-hydroxy fractions of urinary 17-ketosteroid extracts. All depend upon the use of digitonin for precipitation

of the β -fraction. The procedure of Talbot, Butler & MacLachlan (1940a) is not entirely satisfactory in so far as it relies on an indirect determination of the β -fraction. The micro-method of Baumann & Metzger (1940) has been criticized by Salter *et al.* (1946) on the grounds that several conditions (e.g. ethanolic content of the medium, and initial concentration of steroid) may alter the apparent partition between α - and β -fractions. The procedure of Frame (1944) has the single disadvantage that about 15 mg. of total steroid are required, an amount which would necessitate the extraction of several days' collection of urine from subjects with a low 17-ketosteroid excretion. Pincus (1945) described a micro-procedure (using about 1 mg. of total steroid) and suggested the addition of dehydroisoandrosterone (3(β)-hydroxyandrost-5-en-17-one) in order to obtain maximum precipitation of the β -fraction. He also commented that, regardless of the β -17-ketosteroid content, digitonin precipitation leads to a loss of approximately 0.02 mg. of β -17-ketosteroid to the α -fraction. There is evidently a need for a method of separation of α - and β -17-ketosteroids in urine extracts, which would combine the reliability of

Frame's procedure with the convenience of a micro-method. The method described below (based mainly on the methods of Frame, 1944 and of Pincus, 1945) has been developed with these objects in mind. It has been used in the determination of the excretion of α - and β -17-ketosteroids in a series of ten normal men and ten normal women.

EXPERIMENTAL

Collection and treatment of urine

Ten men (medical students and laboratory staff) and ten women (nurses) provided 24 hr. collections of urine. The specimens were collected in vessels containing 7 ml. of conc. HCl, and the importance of accurate collection was stressed. The specimens were kept in a cool place during collection and then stored in a refrigerator until extracted, generally within 24 hr.

The urines were hydrolyzed and extracted according to the method described by Barnett, Henly & Morris (1946), and the extracts were separated into α - and β -fractions by the procedure described below. The final extracts were oxidized by permanganate-periodate, treated with Girard's reagent T and polarographed as described by Barnett, Henly & Morris (1946). The dropping electrode used in the course of this work had the following constants: drop time t , 1.68 sec.; average drop weight, 5.35 mg.; capillary constant $k = m^{\frac{1}{2}} t^{\frac{1}{2}} = 2.36$ (m = mass of mercury dropping/sec.). Androsterone and $3(\beta)$ -hydroxyandrost-5-en-17-one were found to give polarograms with identical wave heights (100 μ g. of each gave a wave height of 17.5 mm. at sensitivity C (cf. Barnett, Henly & Morris, 1946)).

Separation of urinary 17-ketosteroids into α - and β -fractions

Reagents. *Digitonin solution.* A 1% (w/v) solution of digitonin (British Drug Houses, Ltd.) in warm 90% (v/v) ethanol. The digitonin crystallizes from the solution on standing at room temperature, but it may be kept in solution by storing at 37° in a tightly stoppered container. *Ether:* The ether must be peroxide free. Howard's Standard Ether (*Aether Anaestheticus P.B.*) has been found satisfactory. *Pyridine:* The 'Analar' reagent is dried over barium oxide and redistilled at frequent intervals. *Sulphuric acid:* Approx. 2N.

Procedure. A portion of benzene solution of urine extract, containing 1.0–1.5 mg. of 17-ketosteroid, is transferred to a graduated centrifuge tube and evaporated to dryness, preferably under reduced pressure. Warm digitonin solution (0.75 ml.) is added and the mixture quickly heated to boiling (over a microflame) in order to dissolve as much of the material as possible. The tube is stoppered and left in a refrigerator overnight. A total of 10 ml. of peroxide-free ether is then added in small portions, stirring with a thin glass rod between each addition, and allowing the precipitated digitonin and digitonides to flocculate before adding the last 2–3 ml. The mixture is centrifuged for 5 min. at 2000–3000 r.p.m. and the supernatant liquid decanted into a separating funnel of 30–50 ml. capacity. The precipitate in the centrifuge tube is washed three times with 5 ml. portions of ether, stirring and centrifuging each time, as before. The combined supernatant liquids are washed

three times with 5 ml. portions of water, transferred to a small flask, the ether removed under reduced pressure, and the residue dried *in vacuo* over CaCl_2 for about 1 hr. This constitutes the α -fraction.

The residue in the centrifuge tube is dissolved in 0.25 ml. of dry pyridine and warmed in a water bath at 60–70° for 3 min. After cooling in running water, 5 ml. of ether are added in small quantities, stirring between additions and allowing the precipitate to flocculate as before. The mixture is centrifuged for 5 min. and the supernatant liquid poured off into a small separating funnel. The residue in the centrifuge tube is again treated with pyridine and ether, and the final precipitate washed twice with 5 ml. portions of ether. The combined pyridine-ether extracts are washed twice with 5 ml. portions of 2N- H_2SO_4 and three times with 5 ml. portions of water. After transferring to a small flask, the ether is removed under reduced pressure and the residue dried *in vacuo* over CaCl_2 for about an hour. This constitutes the β -fraction.

With normal urines it will usually be necessary to use about one-tenth of the α -fraction and the whole of the β -fraction for oxidation and polarography. In both cases optimal results are obtained when about 60–120 μ g. of 17-ketosteroids are present.

A few experiments have been carried out in which β -fractions obtained from the same urine extract have been polarographed with and without previous oxidation. The results indicate that little improvement may be obtained by oxidation, and further work may show that oxidation of β -fractions is, in fact, unnecessary. It would appear that most of the interfering material removed by oxidation remains with the α -fraction.

RESULTS

Tables 1 and 2 show the results of experiments to test the accuracy of the procedure. In each case the ethanolic solution of pure sterol or urine extract or

Table 1. *Recovery of α - and β -17-ketosteroids from pure solutions*

Exp. no.	α -17-Ketosteroids			β -17-Ketosteroids		
	Added (μ g.)	Recovered		Added (μ g.)	Recovered	
		(μ g.)	(%)		(μ g.)	(%)
1	—	—	—	100	95	95
2	—	—	—	100	100	100
3	—	0	—	200	200	100
4	100	97	97	100	100	100
5	100	100	100	100	100	100
6	100	97	97	200	194	97
7	60	65	108	100	97	97
8	75	71	95	60	61	102
9	200	200	100	88	80	100
10	400	377	94	50	47	94
Means			98.7	98.5		

mixture was evaporated to dryness in a graduated centrifuge tube and separated into α - and β -fractions. Androsterone was used as the α -17-ketosteroid and $3(\beta)$ -hydroxyandrost-5-en-17-one as the β -17-ketosteroid. It will be seen that in all cases the recoveries of both fractions are satisfactory.

Table 2. *Recovery of α - and β -17-ketosteroids from urine extracts*

Exp. no.	α -17-Ketosteroids				β -17-Ketosteroids			
	Present (μ g.)	Added (μ g.)	Recovery of added material		Present (μ g.)	Added (μ g.)	Recovery of added material	
			(μ g.)	(%)			(μ g.)	(%)
11	914	400	401	100	29	100	91	91
12	914	500	516	103	29	50	50	100
13	914	600	571	95	29	150	141	94
14	1255	150	145	97	36	200	193	97
15	1255	0	0	—	36	500	464	93
16	857	279	283	102	57	300	303	101
17	1614	335	356	106	140	400	414	105
			Means	100.5				97.9

Table 3. *The separation of the 17-ketosteroids in a pooled urine extract into α - and β -fractions, at different concentration levels*

Vol. of extract used (ml.)	Total 17-ketosteroids (μ g./ml.)	α -17-Keto-steroids (μ g./ml.)	β -17-Keto-steroids (μ g./ml.)	($\alpha + \beta$)-17-Keto-steroids (μ g./ml.)	($\alpha + \beta$)/total 17-ketosteroids (%)
0.8	1400	1360	82	1442	103.0
1.5	1400	1335	84	1419	101.4
2.5	1400	1300	89	1389	99.2

Table 4. *The urinary excretion of total, α - and β -17-ketosteroids in ten normal men*

Extract no.	Age (yr.)	Volume of urine (ml./24 hr.)	Total 17-ketosteroids (mg./24 hr.)	α -17-Keto-steroids (mg./24 hr.)	β -17-Keto-steroids (mg./24 hr.)	β -17-Keto-steroids (% of total)	Recovery ($\alpha + \beta$)/total 17-ketosteroids (%)
MS 1	20	2260	15.1	12.9	0.8	5.3	91
MS 2	20	2270	13.4	10.6	2.0	14.9	94
MS 3	27	1790	21.8	19.3	1.7	7.8	96
MS 4	24	1850	16.9	15.6	0.6	3.6	96
MS 5	31	1920	10.4	9.6	0.8	7.7	100
MS 6	25	950	19.7	18.3	1.2	6.1	99
MS 7	38	1670	11.9	10.4	0.9	7.6	95
MS 8	32	2600	16.3	14.7	0.3	1.8	92
MS 9	31	1310	11.2	9.4	0.7	6.3	90
MS 10	25	1040	15.9	14.7	0.4	2.5	94
Means			15.3	13.6	0.9	6.4	95
Standard deviations (σ)			± 3.7	± 3.6	± 0.5	± 3.7	± 3
Probable range (mean $\pm 2\sigma$)			7.9-22.7	6.4-20.8	0-1.9	0-13.8	89-101
Standard deviation among duplicates				± 0.5	± 0.03		

Table 5. *The urinary excretion of total, α - and β -17-ketosteroids in ten normal women*

Exp. no.	Age (yr.)	Volume of urine (ml./24 hr.)	Total 17-ketosteroids (mg./24 hr.)	α -17-Keto-steroids (mg./24 hr.)	β -17-Keto-steroids (mg./24 hr.)	β -17-Keto-steroids (% of total)	Recovery ($\alpha + \beta$)/total 17-ketosteroids (%)
WS 1	22	1700	21.4	19.4	1.6	7.5	96
WS 2	20	1200	14.3	13.6	0.6	4.2	99
WS 3	21	1680	10.1	9.4	0.8	7.9	101
WS 4	20	820	8.5	7.7	0.4	4.7	95
WS 5	22	1350	8.5	8.3	0.2	2.4	100
WS 6	18.5	1860	11.9	10.9	0.4	3.4	95
WS 7	21	1250	15.7	15.9	0.4	2.5	104
WS 8	21	775	14.5	13.1	1.0	6.9	97
WS 9	24	710	15.1	14.1	1.0	6.6	100
WS 10	19	2500	20.7	20.2	0.5	2.4	100
Means	20.9	—	14.1	13.3	0.7	4.9	98
Standard deviations (σ)			± 4.5	± 4.3	± 0.4	± 2.1	± 3
Probable range (mean $\pm 2\sigma$)			5.1-23.1	4.7-21.9	0-1.5	0.7-9.1	92-104
Standard deviation among duplicates				± 0.6	± 0.03		

Table 6. *The normal urinary excretion of α - and β -17-ketosteroids as reported by various workers since 1940*

Authors	No. of cases	Sex	α -17-Ketosteroids (mg./24 hr.)				β -17-Ketosteroids (mg./24 hr.)				% of total 17-ketosteroids				Recovery ($\alpha + \beta$)/total 17- ketosteroids		Notes
			Mean	Standard deviation (σ)	Range (mean $\pm 2\sigma$)		Mean	Standard deviation (σ)	Range (mean $\pm 2\sigma$)		Mean	Standard deviation (σ)	Range (mean $\pm 2\sigma$)		Mean (%)	Probable range (%)	
Baumann & Metzger (1940)	12	M.	13.5	3.4	6.7-20.3	0.9	0.9	0-2.7	6.2	5.2	0-16.6	—	—	—	—	(1)*	
Frame (1944)	2	M.	9.2	—	—	0.4	—	—	2.8	—	—	—	—	97	—	(2)	
Salter <i>et al.</i> (1946)	12	M.	13.0	3.3	6.4-19.6	1.8	1.1	0-4.0	10.7	7.3	0-25.3	94	74-114	—	—	(3)	
	12	M.	11.9	3.6	4.7-19.1	1.3	0.5	0.3-2.3	9.5	4.2	0.9-17.9	94	78-110	—	—	(4)	
Butt, Henly & Morris (this paper)	10	M.	13.6	3.6	6.4-20.8	0.9	0.5	0-1.9	6.4	3.7	0-13.8	95	89-101	—	—	(5)	
Baumann & Metzger (1940)	4	F.	4.4	—	—	0.2	—	—	3.8	—	—	—	—	—	—	(6)*	
Frame (1944)	4	F.	6.9	—	—	0.14	—	—	1.75	—	—	—	—	92	—	(7)	
Salter <i>et al.</i> (1946)	13	F.	6.5	2.1	2.3-10.7	1.1	0.9	0-2.9	14.4	11.6	0-37.6	96	63-129	—	—	(8)	
	14	F.	6.5	2.9	0.7-12.3	1.1	0.7	0-2.5	14.9	8.8	0-32.5	96	62-130	—	—	(9)	
Butt <i>et al.</i> (this paper)	10	F.	13.3	4.3	4.7-21.9	0.7	0.4	0-1.5	4.9	2.1	0.7-9.1	98	92-104	—	—	(10)	

* These authors do not give figures for a separate determination of 'total 17-ketosteroids'. The sum of the α - and β -fractions has been used in the calculation of the percentage β -17-ketosteroids.

(1) Ages of subjects were from 17 to 49 yr. (av. 26.3). Most of the results obtained on 3-day urine collections.

(2) Subjects 52 and 62 yr.

(3) *m*-Dinitrobenzene reaction (Cahen & Salter, 1944). On same urine extracts. Subjects aged 20-58 yr. (av. 32.8). Separation into α - and β -fractions carried out by

(4) Antimony trichloride reaction (Pincus, 1943b) } method of Baumann & Metzger (1940).

(5) Polarographic method. Subjects aged 20-38 yr. (av. 27.3).

(6) Subjects aged 26-38 yr. (av. 30). Results obtained on 3-day urine collections.

(7) Subjects aged 23-49 yr. (av. 34.5). Results obtained on 3-day urine collections.

(8) *m*-Dinitrobenzene reaction (Cahen & Salter, 1944). On same urine extracts. Subjects aged 22-54 yr. (av. 30.6). Separation into α - and β -fractions carried out by

(9) Antimony trichloride reaction (Pincus, 1943b) } method of Baumann & Metzger (1940).

(10) Polarographic method. Subjects aged 18.5-24 yr. (av. 20.9).

As a further check on the validity of the method, varying quantities of a pooled urine extract were separated into α - and β -fractions by the routine procedure. The results are shown in Table 3. It will be seen that the partition into α - and β -fractions does not appear to be affected by the initial concentration of steroid (cf. the criticism by Salter *et al.* (1946) of the method of Baumann & Metzger (1940)).

The results of the determination of total, α - and β -17-ketosteroids in the urines of ten normal men and ten normal women are shown in Tables 4 and 5. The values quoted for α - and β -17-ketosteroids are the means of duplicate estimations on the same urine extract. The standard deviations among duplicates indicate that reproducibility is satisfactory. It will be seen that the recovery of 17-ketosteroids after fractionation is also satisfactory. In addition it may be noted that the values for total 17-ketosteroids are within the previously published ranges for normal men and women (Barnett, Henly & Morris, 1946; Barnett, Henly, Morris & Warren, 1946).

Although the mean values for the excretion of β -17-ketosteroids are greater for men than for women, both when measured in terms of mg./24 hr. and as percentage of the total ketosteroids, application of Fisher's 't' test shows that the differences are not statistically significant.

DISCUSSION

The results of some recent work on the determination of α - and β -17-ketosteroids in normal individuals are collected in Table 6. The data given by the various workers have been used in the calculation of means, standard deviations, etc. In addition the following authors have indicated normal ranges for β -17-ketosteroid excretion without giving individual values: β -fraction usually at least 10–15% of the total 17-ketosteroids (Talbot, Butler & MacLachlan, 1940b); β -fraction is 0.6–2.2% of total 17-ketosteroids (Dobriner *et al.* 1942); β -fraction seldom exceeds 15% and is usually 2–3% of the total 17-ketosteroids (Pincus, 1943a).

It will be seen from Table 6 that our results for the excretion of α -17-ketosteroids by men are in moderately good agreement with those of other workers. In the case of the women, however, the values found are considerably higher than those found previously. It may be noted that Barnett, Henly, Morris & Warren (1946) found that the polarographic method gave similarly higher results for total 17-ketosteroids, particularly in women.

Our values for β -17-ketosteroid excretion by men show general agreement with those of Baumann &

Metzger (1940), but the results obtained by Salter *et al.* (1946) are slightly higher. The corresponding values for women show that the results of Salter *et al.* (1946) are again high, the difference being most marked when the results are expressed as percentage of the total 17-ketosteroids. Some of the normal subjects studied by Salter *et al.* (1946) excreted very large quantities of β -17-ketosteroids. One subject (A.M.) excreted 3.9 mg./24 hr., equivalent to 45.6% of the total 17-ketosteroids, though it may be noted that in this case the sum of the α - and β -17-ketosteroids was some 25% greater than the directly determined value for total 17-ketosteroids (results by *m*-dinitrobenzene reaction).

While each author quoted obtained satisfactory mean recoveries of 17-ketosteroid after fractionation of urine extracts, it is interesting to note the very wide range of recoveries obtained by Salter *et al.* (1946). The reason for this inconsistency is not clear.

Finally, the results described in this paper indicate that the β -17-ketosteroids may contribute up to 15% to the total 17-ketosteroid excretion of normal individuals, though values less than 10% are more usual. No certain sex difference can be demonstrated at the present time. This conclusion is in general agreement with the findings of Baumann & Metzger (1940), Dobriner *et al.* (1942) and Pincus (1943a), but is only in partial agreement with the values quoted by Talbot *et al.* (1940b).

SUMMARY

1. A micro-method has been described for the quantitative separation of urinary 17-ketosteroid extracts into 3(α)- and 3(β)-hydroxy fractions. The 17-ketosteroids in the separated fractions are estimated polarographically.
2. Recovery of α - and β -17-ketosteroids from pure solutions and from urine extracts is satisfactory.
3. The urinary excretion of α - and β -17-ketosteroids has been determined in a series of ten normal men and ten normal women, and the results have been compared with those obtained by other workers.
4. It is concluded that the total 17-ketosteroids excreted by normal individuals may contain up to 15% of 3(β)-hydroxy compounds, though values less than 10% are more general. There is no significant difference between the β -17-ketosteroid excretion of men and women.

The authors wish to express their thanks to the men and women who co-operated in the collection of 24 hr. urine specimens.

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Studies in Human Mineral Metabolism

1. THE EFFECT OF BREAD RICH IN PHYTATE PHOSPHORUS ON THE METABOLISM OF CERTAIN MINERAL SALTS WITH SPECIAL REFERENCE TO CALCIUM

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The discovery that phytic acid forms relatively insoluble salts with several of the common bases, that are of importance in our diet, has led to several investigations in which the availability of these bases has been determined with varying intakes of phytic acid. The conclusions drawn from such work have been somewhat contradictory: thus, whilst all observers are agreed that the addition of a considerable amount of phytate P is invariably followed by an immediate reduction in the amount of Ca retained, some investigators have suspected, and others are emphatic, that, given time, an adaptation to such a change can occur, and that eventually the body is able to adjust itself fairly satisfactorily to the new conditions.

Obviously laboratory investigations must eventually be reconciled with what happens in everyday life, and a consideration of those human diets which are rich in cereals and hence in phytic acid, yet poor in Ca, lends a considerable amount of support to the view that some such process of adaptation must in fact take place. Otherwise it is difficult to explain how the consumers avoid the disastrous consequences which would arise if part or the whole of this Ca were rendered unavailable.

For two reasons this issue is of particular importance to a country like South Africa. In the first place the large majority of the population, including most of the non-Europeans and not a few of the poorer Europeans, live on just such a diet as has been mentioned above. Secondly, in 1941, a compulsory Standard War Bread, made from 95 to 100 % extraction meal, was introduced and is still the only kind of bread allowed to be baked.

As a result of the work published by McCance & Widdowson (1942), the South African National Nutrition Council decided that it was imperative to study the problem under local conditions. For this purpose balance experiments, similar to those described by these workers, were initiated to observe the effects of breads made from high and low extraction meals upon mineral metabolism in adult male subjects.

EXPERIMENTAL

Plan of the experiments. The intake and excretion of Ca, Mg, total P, phytate P and Fe, were determined on four healthy adult European males, over periods ranging from 3 to 19 consecutive weeks.