REFERENCES

Bamberger, E. & Pyman, F. L. (1909). *Ber. dtsch. chem. Ges.* **42**, 2310.
Baumann, E. & Preusse, C. (1879). *Hoppe-Seyl. Z.* **3**, 156.
Bratton, A. C. & Marshall, J. K. Jr. (1939). *J. biol. Chem.* **128**, 537.
Bray, H. G., James, S. P., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **42**, 274.
Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946*a*). *Biochem. J.* **40**, 134.
Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946*b*). *Biochem. J.* **40**, 406.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1947). *Biochem. J.* **41**, 212.
Bruce, H. M. & Parkes, A. S. (1946). *J. Hyg., Camb.*, **44**, 501.
Eckert, H. W. (1943). *J. biol. Chem.* **148**, 197.
Einhorn, A. & Pfyl, B. (1900). *Liebigs Ann.* **311**, 43.
Ellinger, A. & Hensel, M. (1914). *Hoppe-Seyl. Z.* **91**, 21.
Fildes, P. (1940). *Lancet*, **1**, 955.
Folin, O. (1905–6). *J. biol. Chem.* **1**, 131.
Friedländer, P. (1916). *Ber. dtsch. chem. Ges.* **49**, 964.
Griess, P. (1878). *Ber. dtsch. chem. Ges.* **11**, 1734.
Griess, P. (1887). *Ber. dtsch. chem. Ges.* **20**, 407.
Harrow, B., Mazur, A. & Sherwin, C. P. (1933). *J. biol. Chem.* **102**, 35.
Hensel, M. (1914). *Hoppe-Seyl. Z.* **93**, 401.
Hildebrandt, H. (1903). *Beitr. chem. Physiol. Path.* **3**, 365.
Hirsch, J. (1942). *Science*, **96**, 139.
Horn, F. (1936*a*). *Hoppe-Seyl. Z.* **238**, 84.
Horn, F. (1936*b*). *Hoppe-Seyl. Z.* **242**, 23.
Jacobs, W. A. & Heidelberger, M. (1917). *J. Amer. chem. Soc.* **39**, 1437.
Jaffe, M. & Hilbert, P. (1888). *Hoppe-Seyl. Z.* **12**, 295.
Kaiser, A. (1885). *Ber. dtsch. chem. Ges.* **18**, 2946.
Kalle and Co. (1911). D.R.-P. 232, 277; *Chem. Zbl.* **1**, 1019.
Keller, O. (1908). *Arch. Pharm., Berl.*, **246**, 1.
Kleine, F. K. (1896–7). *Hoppe-Seyl. Z.* **22**, 327.
Kohl, M. F. F. & Flynn, L. M. (1941). *Proc. Soc. exp. Biol., N.Y.*, **47**, 470.
Kossel, A. (1880). *Hoppe-Seyl. Z.* **4**, 296.
Limpricht, H. (1891). *Liebigs Ann.* **263**; 234.
Meldola, R., Foster, H. S. & Brightman, R. (1917). *J. chem. Soc.* **111**, 533.
Mitsuba, K. & Ichihara, K. (1927). *Hoppe-Seyl. Z.* **164**, 244.
Muenzen, J. B., Cerecedo, L. R. & Sherwin, C. P. (1926). *J. biol. Chem.* **67**, 469.
Quick, A. J. (1932). *J. biol. Chem.* **96**, 83.
Salkowski, E. (1872). *Ber. dtsch. chem. Ges.* **5**, 637.
Salkowski, E. (1873). *Ber. dtsch. chem. Ges.* **6**, 744.
Salkowski, E. (1882). *Hoppe-Seyl. Z.* **7**, 93.
Schmidt, C. L. A. & Allen, E. G. (1920). *J. biol. Chem.* **42**, 55.
Sieburg, E. (1914). *Hoppe-Seyl. Z.* **92**, 331.
Stubbs, A. L. & Williams, R. T. (1947). *Biochem. J.* **41**, xlix.
Ville, J. (1892). *C.R. Acad. Sci., Paris*, **114**, 228.
Woods, D. D. (1940). *Brit. J. exp. Path.* **21**, 74.
Zahn, O. (1900). *J. prakt. Chem.* (2), **61**, 532.
Zehender, F. (1943). *Helv. chim. Acta*, **26**, 1338.

Separation of Acidic Amino-acids by means of a Synthetic Anion Exchange Resin

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Synthetic ion-exchange resins have recently been widely used for the separation of basic and acidic from neutral compounds. Thus Cannan (1944), by repeated applications of Amberlite IR 4, separated glutamic and aspartic acids quantitatively from protein hydrolysates. Earlier, Freudenberg, Walch & Molter (1942) had used a column of Wolfatite M for the separation of the dicarboxylic amino-acids, and Cleaver, Hardy & Cassidy (1945) investigated the chromatographic separation of amino-acids by organic exchange resins. Recently, Tiselius, Drake & Hagdahl (1947) have employed synthetic ion exchange resins chromatographically for the quantitative separation of amino-acids into groups. The aim of the present investigation was to extend such methods to cover the separation of the acidic amino-acids from one another (at pH 2·5) after their isolation from the rest of the protein hydrolysate (at pH 3–4). Although completely quantitative recoveries were not obtained, complete separations have been achieved. The separation depends on differences in net charge between the acidic amino-acids at a given pH (Fig. 1). Thus, glutamic acid with pK_1 2·16 and pK_2 4·32 (Neuberger, 1936) has a net charge of −0·31 at pH 4; aspartic acid with pK_1 1·88 and pK_2 3·65 (Miyamoto & Schmidt, 1931) has a net charge of −0·69 at pH 4; whilst cysteic acid with pK_1 1·88 and pK_2 1·30 (Andrews & Schmidt, 1927)

has a net charge of −0·99 at this pH. It would therefore be expected that a column of Amberlite IR4 maintained uniformly at pH 4, because of its positive charge, will preferentially retain cysteic, aspartic and glutamic acids in this order. Theoretically, it would appear that the optimum pH for the separation of glutamic from aspartic acid is about 4, since at this pH the largest difference in net charge occurs, but experimentally it was found that for satisfactory separation a pH of 2·5 was necessary. At this pH glutamic and aspartic acids normally carry a net positive charge of +0·29 and +0·13 respectively. Nevertheless, at this pH they are retained on Amberlite IR4 columns and can be separated by elution in relatively small volumes; at higher pH values, although separation will take place, relatively large volumes are required. This suggests that the pK values are depressed when these amino-acids are adsorbed on Amberlite IR4. Similarly, cysteic acid is partially retained at pH 1·5, although its net charge at this pH should be almost zero.

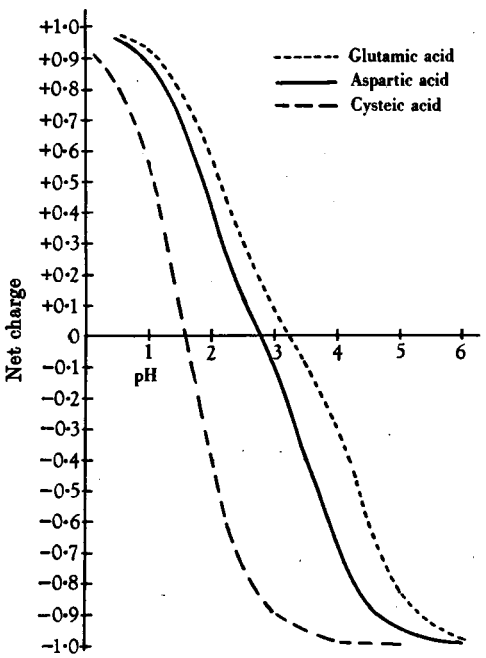

Fig. 1. Net charge of glutamic, aspartic and cysteic acids at different pH values, calculated from the dissociation constants given in the text.

To apply these principles, care has been taken to bring the Amberlite to the required pH and to maintain it uniformly in that part of the column where separations are taking place. In order to establish equilibrium conditions, the resin was finely powdered and the width of the column kept small in relation to its height so that low rates of flow were maintained. In the first stage, no difficulty was found in effecting the separation of the acidic from the basic and neutral amino-acids in a protein hydrolysate at pH 3–4. As is shown by its titration curve (Fig. 2), at this rather acid pH, the percentage charge on the resin is sufficiently high for it to compete successfully with lysine and arginine for the acidic amino-acids. The departure from the expected sigmoid shape of the curve below pH 1 may be due to decomposition of the resin. This is also suggested by the fact that the nitrogen (Kjeldahl) washed from a column by acid is about ten times as high for strong acid (pH 0–1) as for weak acid (pH 3–4) and is independent of the history (previous washings) of the column. By the above means and by the application of the chromatographic principle, considerable economy of resin was achieved. Separations have been followed by partition chromatography on paper (Consden, Gordon & Martin, 1944) of samples from each eluate; this method has provided a convenient means for identifying the amino-acids present in each fraction, without using more than negligible quantities.

The copper titration method of Pope & Stevens (1939) was chosen as a convenient means of estimating the acidic amino-acids after separation by the resin and was adapted to deal with micro quantities. Unfortunately, it was not found possible to obtain fully quantitative recoveries of glutamic and aspartic acids after their separation from synthetic mixtures. The recovery of glutamic acid was about 91%, that for aspartic about 96%. A possible cause for low recoveries may be formaldehyde which appears to be continually liberated even after the washing cycle employed by Cannan (1944). Attempted removal of formaldehyde failed to improve the results. Estimation of amino N or total N in the eluates by the Van Slyke manometric procedure or Kjeldahl method was tried and abandoned owing to the high blanks given by eluates washed off the column after the removal of amino-acids. Cysteic acid, as isolated from hydrolysates by means of Amberlite, is always accompanied by pigment having a similar acid strength. As similar pigment material from hydrolysates of wool not containing cysteic acid gives significant titrations in the Pope & Stevens (1939) procedure, estimations of cysteic acid would presumably give fictitiously high results.

As the synthetic ion exchange resins can absorb relatively large amounts of amino-acids (Englis & Fiess, 1944; Cleaver *et al.* 1945), they are suitable for isolations. Thus, cysteic acid has been isolated from a hydrolysate of chlorinated wool. This material was almost fully optically active, whereas that obtained by Lissizin (1928) from hair oxidized by alkaline permanganate was inactive.

METHODS

*Preparation of the Amberlite IR4.** The resin is alternately ground wet in a ball mill and sieved with a jet of water, −90 +200 grading being used for the columns. It is washed free from NH_3, treated with HCl until the supernatant liquor is permanently acid to thymol blue (pH <1) and then washed with water. Alternate treatments with acid and water are repeated a number of times.

Preparation of columns. (i) The resin is stirred and washed by decantation with water until the supernatant liquid is less than 0·001 N with respect to alkali. The slurry is transferred to a glass tube of 0·5 cm. internal diameter, forming a column 20 cm. in height (containing about 2 g. air-dry resin), and washed overnight with water at a rate of approx. 15 ml./hr. This does not significantly raise the pH of the eluate, the acid concentration of which should be checked by titration before the next stage. (ii) The second column (height 30 cm.) is prepared from about 3 g. Amberlite and the pH is adjusted accurately to 2·5 by equilibration with dilute acid or water until the acidity of the supernatant fluid is slightly above 0·003 N. The material is then transferred to a 0·5 cm. diameter tube and washed overnight with 0·003 N-HCl. The acidity of the eluate is finally checked by titration. The columns should not be allowed to run dry and remain so for any length of time; otherwise extensive cracking may occur.

Procedure for estimation of glutamic and aspartic acids. The hydrolysate prepared with HCl and containing up to 15 mg. acidic amino-acids is repeatedly evaporated *in vacuo* by rotating manually in a warm water bath, allowed to stand overnight *in vacuo* over KOH, and quantitatively transferred in a small volume to the first column. Water is then passed down the column; the neutral and basic amino-acids are eluted in the first 25 ml. The next 5 ml. should be free from amino-acids. To test the eluates, a portion of the first 25 ml. and the whole of the second 5 ml. fraction are separately concentrated *in vacuo* to very small bulk and samples of each tested by partition chromatography on a strip of Whatman no. 4 paper using phenol-NH_3, developing for 5 hr. or more, with suitable control (cf. Consden *et al.* 1944). After development and treatment with ninhydrin, basic and neutral, but no acidic amino-acids, are found in the first eluate, whilst the second eluate should contain no amino-acids. If cystine is present, the eluate sample is oxidized by treatment with excess Br_2 water before chromatography; the cysteic acid thus produced occupies a position on the chromatogram not overlapping the positions of aspartic and glutamic acids (Consden, Gordon & Martin, 1946) if these are present.

The next stage consists in transferring the material remaining in the column to the second column. This may be done in either of two ways:

Method 1. The column is eluted with N-HCl causing the pigment to run down as a sharp band. When this has run out, the washing is stopped and the eluate repeatedly evaporated *in vacuo* and allowed to stand overnight *in vacuo* over KOH to remove excess HCl. It is then quantitatively transferred to the second column which is eluted with 0·003 N-HCl, 10 ml. eluates being collected. Each is concentrated *in vacuo* to a volume of 1–2 ml. and a sample (4 μl.) tested as described above.

* Obtained from the Resinous Products and Chemical Co., Pa.

Method 2. The column is washed with 3 ml. N-HCl, causing the pigment band to travel about half way down. (The eluate should be free from amino-acids as revealed by chromatography.) The column is then fixed to the top of the second column. Elution is continued with water, but the acid already added is sufficient to cause the pigment band to continue to travel down and eventually to pass into the lower column. When the pigment has travelled a few cm. into the second column, the top column is removed and elution of the lower column continued with 0·003 N-HCl. If no pigment is present, elution may be followed by the change in shade of the resin which is orange when strongly acid. Eluates are collected and tested as in method 1.

Method 1 has been found to behave more consistently than method 2; in proteins where the ratio of glutamic to aspartic acid is of the order 2 : 1, the glutamic acid is eluted from the second column in the first 20 ml., and aspartic acid in the next 30 ml., whereas in method 2 the stages at which glutamic and then aspartic acids begin to appear are rather less certain. Method 2 is quicker than method 1 but requires more attention.

Each eluate is evaporated as above *in vacuo* to dryness, and a known volume (5–10 ml.) of copper phosphate-buffer suspension (Pope & Stevens, 1939) is added to the crystalline residue. Titrations (20–50 μl. 0·1 N-$Na_2S_2O_3$) are carried out on 1 ml. portions after filtering and addition of the other reagents. Neutralization before addition of the copper phosphate is unnecessary.

Procedure for the isolation of cysteic acid. In order to assess the amount of protein from which a workable quantity of cysteic acid may be isolated, its amount in a hydrolysate is roughly estimated by partition chromatography on paper. A known volume (5–10 μl.) is applied to Whatman no. 4 paper and known amounts (0·5–20 μg.) of cysteic acid, each at the same volume as that of the hydrolysate, applied by the side of the hydrolysate spot. After development of the chromatogram in phenol-NH_3-coal gas for 17 hr., the paper is dried, coloured with ninhydrin and the depth of colour of the cysteic acid spot in the hydrolysate compared with those of the controls. The isolation may be conducted either (*a*) by eluting the column with N-HCl after separation of the dicarboxylic amino-acids by method 1 or 2, or (*b*) at pH 1·5. If (*a*) has been used, partition chromatography reveals that the whole of the cysteic acid runs out in the region of the pigment band, at which stage there is a sharp change in pH from 2·5 to <1. No complete separation of the pigment from cysteic acid was achieved by using the resin and eluting solution at a lower pH; however, at pH 1·5 all the amino-acids except cysteic acid are rapidly eluted, thus making possible its isolation on only one column. Thus, on a column, length 30 cm., diameter 0·5 cm., eluted with 0·03 N-HCl, the neutral, basic and dicarboxylic amino-acids accompanied by some pigment, run out in the first 25 ml. followed soon after by cysteic acid, the elution of which can be speeded up by N-HCl.

RESULTS

Recovery of glutamic and aspartic acids from a mixture of amino-acids

An amino-acid mixture simulating a wool hydrolysate (Astbury, 1942) was prepared containing 0·0517 mg. aspartic acid N (4·12% of total N) and

Table 1. *Recoveries of glutamic and aspartic acids from a synthetic mixture*

		Experiment							
		(i)		(ii)		(iii)		(iv)	
	Amount (mg. N)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)
Glutamic acid	0·8416	0·767	91·3	0·773	92·0	0·776	92·3	0·747	88·8
Aspartic acid	0·4126	0·394	95·2	0·390	94·2	0·404	97·6	0·397	96·0

0·1052 mg. glutamic acid N (8·40 % of total N)/ml. This solution (8 ml.), after evaporation *in vacuo*, was subjected to the foregoing procedure, using method 2. Recoveries of glutamic and aspartic acids are recorded in Table 1.

In experiments on glutamic and aspartic acids alone, similar recoveries were obtained. The odour of formaldehyde could usually be detected in the eluates from the columns after concentration; treatment of the eluates with alkali, HCl, H_2O_2, or dimedone failed to improve the results and treatment with strong NH_3 gave high results. Addition of formaldehyde to glutamic and aspartic acid solutions led to low titrations.

Isolation of cysteic acid

Degraded protein produced by the action of chlorine on wool (Consden *et al.* 1946), containing 156 mg. N and which by the paper chromatographic method described above was expected to contain approx. 4 mg. cysteic acid N, was hydrolyzed and prepared as already described for application to the resin. This material was transferred to a column (30 cm. high; 1·5 cm. diam.) which had been equilibrated to pH 1·5 and was then eluted with 0·03 N-HCl, the rate of flow through the column being about 60 ml./hr. Six eluates of 20 ml. each were collected and tested as described above. The neutral amino-acids plus glutamic and aspartic acids were eluted in the first three fractions. The fourth fraction contained much cysteic acid and subsequent fractions successively less. The column was eluted with a further 500 ml. 0·03 N-HCl and, as small amounts of cysteic acid were still being washed out, it was finally eluted with N-HCl which removed all the remaining cysteic acid and yellow material from the resin. The united fractions containing cysteic acid were concentrated *in vacuo* to small volume and the solution, after standing at 0°, deposited brownish crystals, which after filtration were decolorized by boiling with animal charcoal. The mixture was filtered, evaporated *in vacuo* to dryness and recrystallized three times from aqueous ethanol. The pale yellow powder (15 mg.) had $[\alpha]_D^{18°} + 7·1°$ (*l.* 2; $c = 0·3$) in water. Clarke & Inouye (1931) give $[\alpha]_D^{24·5°} + 9·4°$ for L-cysteic acid. Its equivalent by titration with $Ba(OH)_2$ was 185; calc. for $C_3H_7O_5N.H_2O$, 187; mixed partition chromatograms using phenol-NH_3-coal gas showed no difference from authentic L-cysteic acid. Analysis of the copper salt gave Cu 25·78; calc. for $C_3H_7O_6NSCu$, Cu, 25·57 %.

Titration of Amberlite IR 4

The resin, which had been treated as described at the beginning of the experimental section, was brought to *c.* pH 10 by treatment with dilute NaOH. It was then thoroughly washed with water and left to dry at room temperature. A sample of the resin (202·8 mg.) on drying to constant weight at 105° lost 30·4 mg. Samples of air-dry resin (200 mg.) were mixed with varying quantities of HCl and each made up to a final volume of 20 ml. and allowed to equilibrate overnight. The pH of the filtrate was determined (glass electrode) and acidity by titration against alkali. The absorption of acid by Amberlite IR 4 at different pH values is shown in Fig. 2.

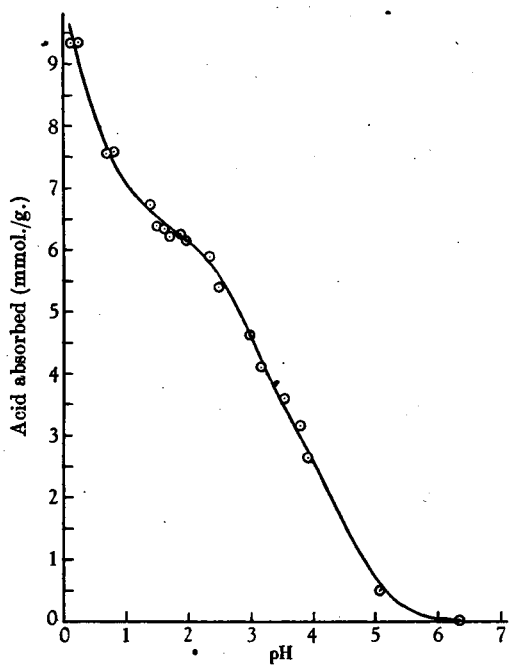

Fig. 2. Titration curve of Amberlite IR4.

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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REFERENCES

Andrews, S. & Schmidt, C. L. A. (1927). *J. biol. Chem.* **73**, 651.
Astbury, W. T. (1942). *J. chem. Soc.* p. 338.
Cannan, R. K. (1944). *J. biol. Chem.* **152**, 401.
Clarke, H. T. & Inouye, J. M. (1931). *J. biol. Chem.* **94**, 541.
Cleaver, C. S., Hardy, R. A. & Cassidy, H. G. (1945). *J. Amer. chem. Soc.* **67**, 1343.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1946). *Biochem. J.* **40**, 580.
Englis, D. T. & Fiess, H. A. (1944). *Industr. Engng Chem.* **36**, 604.
Freudenberg, K., Walch, H. & Molter, H. (1942). *Naturwissenschaften*, **30**, 87.
Lissizin, T. (1928). *Hoppe-Seyl. Z.* **173**, 309.
Miyamoto, S. & Schmidt, C. L. A. (1931). *J. biol. Chem.* **90**, 165.
Neuberger, A. (1936). *Biochem. J.* **30**, 2085.
Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
Tiselius, A., Drake, B. & Hagdahl, L. (1947). *Experientia*, **3**, 21.

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 (1940*a*) 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 dehydro*iso*androsterone (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