PAPER CHROMATOGRAPHY OF URINARY INDOLES EXTRACTED UNDER ALKALINE AND ACID CONDITIONS*

HERBERT SPRINCE, CLARENCE PARKER, JOHN T. DAWSON, JR., DOROTHY JAMESON AND F. CURTIS DOHAN

Laboratory of Research Biochemistry, Veterans Administration Hospital, Coatesville, Pa.

and

Departments of Psychiatry and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pa. (U.S.A.)

(Received December 8th, 1961)

In recent years, a disturbed indole metabolism has been implicated in carcinoid disease[1] and in certain diseases with mental symptoms[2] (*e.g.* phenylketonuria, Hartnup Disease, pellagra, and possibly schizophrenia). This has stimulated a number of publications dealing with paper chromatographic methods for urinary indoles. These methods have involved a variety of procedures with urine, generally utilizing the following techniques: (1) intact urine, unconcentrated[3], or concentrated *in vacuo* 4 to 5 fold[4]; (2) adsorption on both deactivated[5,6], or activated (untreated)[7] carbon followed by elution with aqueous phenol; (3) adsorption on untreated carbon followed by elution with a mixture of ammonia–methanol–butanol–water[8,9]; (4) extraction with ethyl acetate at pH 1–2 after saturation with NaCl[10,11]; and (5) extraction with ethyl ether first at pH 8.5–9.0 (ammonia), next at pH 4.0 (dilute HCl), followed by pooling of ether extracts[12]. Each of these procedures has its own strong and weak points. Thus far, however, there has been no comparison of the effect of alkaline *vs.* acidic conditions on the extraction of urinary indoles by a series of selective solvents used sequentially. Such a study would be valuable in revealing optimal conditions for fractionating urinary indoles. This should permit the eventual development of better methods of quantitation of the various indoles.

Previous reports[13–16] from this laboratory have outlined a method for the differential extraction of urinary indoles under alkaline conditions first with ethyl ether followed by 2-butanone. It is the purpose of this paper: (a) to compare the effect of alkaline *vs.* acidic conditions on the extraction of indoles from urine by the ether–butanone method, (b) to study the effect of tryptophan load on the pattern of indole excretion by this method, and (c) to examine the effect of pretreatment of urine by acid, alkaline, or enzymic hydrolysis on the extraction of indoles by this method under alkaline and acidic conditions.

* The work presented in this paper was aided in part by Research Grants M-1015 (C2) and M-1015 (C3) from the National Institute of Mental Health, National Institutes of Health, U.S. Public Health Service, Bethesda, Md.

EXPERIMENTAL

Fasting overnight urines were collected from normal male subjects (25–50 years of age) and the following data were obtained: name, date, specific gravity, total urine volume, and time interval of collection. In several instances, overnight urines from 4 subjects or more were pooled. Since all chromatographic data were to be expressed in terms of a urinary creatinine equivalent rather than on a 24 hour basis, a creatinine determination was made on each urine or urine pool by the alkaline picrate method and expressed as milligrams creatinine per milliliter of urine. When storage was necessary, toluene was added, and the urines were placed in the deep freeze (—15°) until ready for use.

A measured volume (200–300 ml) of urine generally ranging in specific gravity from 1.010–1.030 and in creatinine concentration from 0.8–2.0 mg/ml was concentrated *in vacuo* with mild heat (37–40°) to a convenient working volume generally containing a creatinine concentration of about 10 to 20 mg/ml (*i.e.* 10–20 fold concentration). The concentrate was then centrifuged and filtered and the residue washed with distilled H_2O (3 × 1/10 volume of filtrate). The filtrate and washings were pooled, saturated with crystalline $(NH_4)_2SO_4$, again filtered, and washed with 2–4 ml of saturated $(NH_4)_2SO_4$ solution in water. This filtrate and washings were again pooled, the total volume measured and divided into two equal volume aliquots, each aliquot representing one-half the original urine volume.

The first aliquot was subjected to alkaline extraction by adjusting to pH 10 with 40 % NaOH dropwise and noting the resulting volume. This solution was then extracted four times with equal volumes of ethyl ether and then four times with equal volumes of 2-butanone. The second aliquot was subjected to an acidic extraction in a similar fashion after adjustment to pH 2 with concentrated HCl. Thus four extracts were obtained: (a) alkaline ethyl ether extract, (b) alkaline 2-butanone extract, (c) acidic ethyl ether extract and (d) acidic 2-butanone extract. All four extracts were then individually evaporated to dryness *in vacuo* and made up in 95 % ethanol to a volume equivalent to 100 mg of urinary creatinine per ml of extract. For chromatography, 0.3 ml of the ethanol extract (═◇═ 30 mg urinary creatinine) were applied to $18^1/_4$ in. × $22^1/_2$ in. sheets of Whatman No. 1 Chromatography Paper. It is important to use sheets of this size for extracts containing 30 mg urinary creatinine equivalent. Papers were then subjected to two-dimensional descending chromatography, using first an isopropanol (I)–ammonium hydroxide (AH)–water (W) system in ratio of 8:1:1, followed by an *n*-butanol (B)–acetic acid (AA)–water (W) system in ratio of 4:1:5. Chromatograms were sprayed with a modified Ehrlich benzaldehyde reagent (EBR) which consisted of 2 % *p*-dimethylaminobenzaldehyde (w/v) dissolved in concentrated HCl (s.g. = 1.19), followed by a 1 % solution of $NaNO_2$ (w/v) in H_2O. Indole compounds with the exception of indican developed blue colors immediately after the nitrite spray and were stable[14].

Typical chromatograms obtained from normal male urines are depicted in Figs. 1 and 2 in the following section.

Paper chromatograms were also obtained by the above procedure on intact urine or urine concentrates (approx. 10 fold) subjected to acid, alkaline, or enzymic hydrolysis. For *acid hydrolysis*, 200–300 ml of intact urine (or equivalent urine concentrate) were brought to 1 N HCl concentration with 10 *N* HCl, refluxed for 15 minutes

at the boiling point, cooled, and adjusted to pH 7 with 10 *N* NaOH. With intact urine, this solution was now concentrated 10 fold and filtered; this was not necessary for urine concentrates. The solution was now saturated with $(NH_4)_2SO_4$, filtered, divided into two equal aliquots, and subjected to alkaline and acid extraction with ether and butanone as described above. For *alkaline hydrolysis*, the same procedure as for acid hydrolysis was used except that, conversely, adjustment to 1 *N* NaOH concentration was made with 10 *N* NaOH and final adjustment to pH 7 was made with 10 *N* HCl. For *β-glucuronidase hydrolysis*, 200–300 ml of intact urine (or equivalent urine concentrate) were adjusted to pH 4.5 with acetic acid–sodium acetate buffer. One hundred units of β-glucuronidase (Warner Chilcott's Ketodase or Worthington's β-Glucuronidase) for each ml of intact urine were added. The solution was layered with toluene, shaken gently, and incubated for 24 hours at 37°. After this time, the solution was adjusted to pH 7, concentrated 10 fold and filtered if intact urine was used, saturated with $(NH_4)_2SO_4$ and again filtered, divided into two equal aliquots, and subjected to alkaline and acid extraction with ether and butanone as described above.

RESULTS

Typical chromatograms obtained from a total of 50 normal male urines (individual or pooled) after alkaline and acidic extractions are depicted in Figs. 1 and 2.

For each chromatogram, 0.3 ml of ethanol extract (=<>= 30 mg urinary creatinine) was used. Only those spots which were well-defined soon after spraying and occurred with marked regularity in most of the urines tested are presented. Generally, each spot represents about 50–150 μg of a given indolic compound except in the case of indican or tryptophan where the value may be much higher. Occasionally, other spots

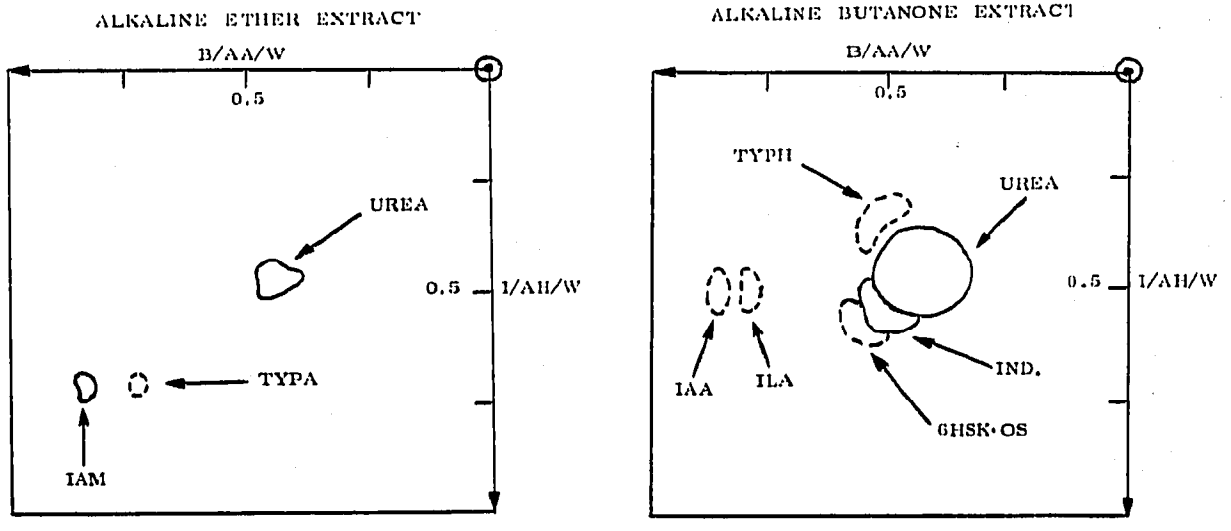

Fig. 1. Two dimensional descending chromatograms of alkaline urine extracts (sprayed with 2% EBR–1% $NaNO_2$). Spots in *solid* lines are generally found in normal male urines. Spots in *dotted* lines are found occasionally or only under certain conditions (see text). Abbreviations are as follows: (figures in parentheses are R_F values in I/AH/W and B/AA/W, respectively), IAM = indoleacetamide (0.70, 0.85); TYPA = tryptamine (0.71, 0.72), not normally found; TYPH = tryptophan (0.28, 0.56); IND. = indican (0.56, 0.53); 6 HSK·OS = 6-hydroxyskatole sulfate (0.60, 0.64); IAA = indoleacetic acid, probably as an ammonium salt (0.50, 0.88); and ILA = indolelactic acid, probably as an ammonium salt (0.48, 0.82). Urea has R_F values of 0.46, 0.45.

appeared after prolonged standing, but they were often ill-defined and not consistently observed. These are not shown in Figs. 1 and 2. Identification of spots was accomplished by comparison of R_F values with synthetic compounds*, co-chromatography

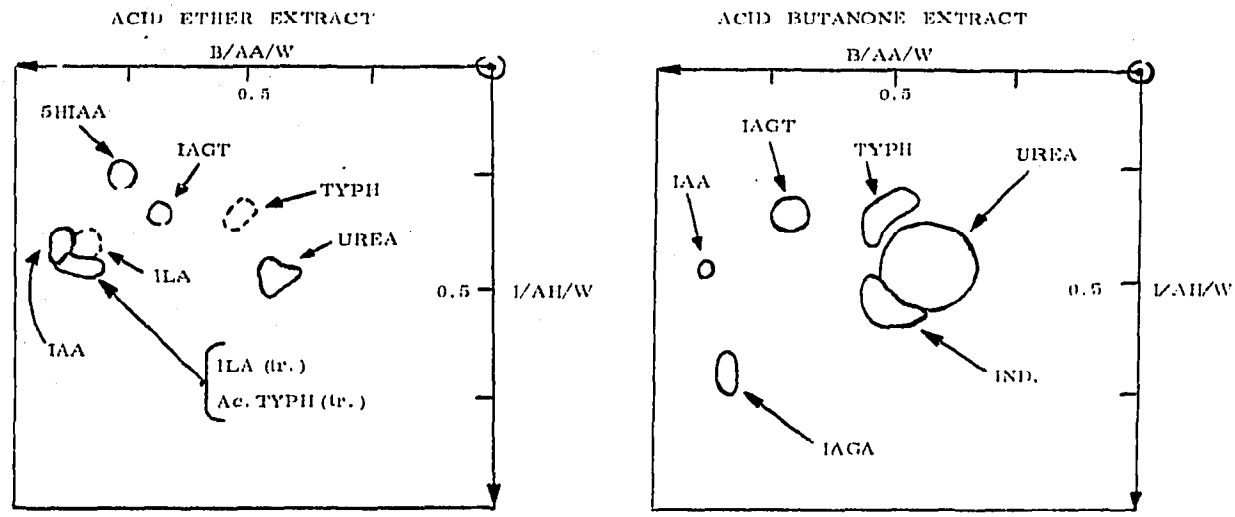

Fig. 2. Two dimensional descending chromatograms of acid urine extracts (sprayed with 2% EBR–1% $NaNO_2$). Spots in *solid* lines are generally found in normal male urines. Spots in *dotted* lines are found occasionally or only under certain conditions (see text). Abbreviations are as follows: (figures in parentheses are R_F values in I/AH/W and B/AA/W, respectively), 5 HIAA = 5-hydroxyindoleacetic acid (0.25, 0.78); IAGT = indoleacetylglutamine (0.31, 0.72); TYPH = tryptophan (0.30, 0.58); IAA = indoleacetic acid (0.42, 0.90); ILA = indolelactic acid (0.40, 0.83); Ac·TYPH = N-acetyltryptophan (0.48, 0.84); IND. = indican (0.56, 0.53); IAGA = indoleacetylglucuronic acid (0.68, 0.85). (See ADDENDUM.) Urea has R_F values of 0.41, 0.43.

of urine extracts with synthetic compounds, utilization of differential solvent systems, and utilization of differential spraying reagents as outlined by JEPSON[17]. R_F values in this paper are presented in the following order with respect to solvent systems: *first value* is for I/AH/W; *second*, for B/AA/W.

A. Alkaline ethyl ether extract (pH 10)

The alkaline ether extract consistently showed two well-defined spots with EBR–nitrite spray in all urines tested. These were: (a) a yellow urea spot with R_F = 0.46, 0.45 and (b) a deep-blue spot with R_F = 0.70, 0.85, respectively. The deep-blue spot was identified as indoleacetamide (IAM) by SPRINCE *et al.*[15]. Marked reduction or disappearance of the IAM spot could be effected: (a) by replacing $(NH_4)_2SO_4$ with NaCl or Na_2SO_4 during the extraction procedure or (b) by pretreating the intact urine, or 10-fold urine concentrate, with β-glucuronidase before saturation with $(NH_4)_2SO_4$ and subsequent extraction at pH 10. These observations would indicate

* Indole compounds used for identification purposes were obtained from the following sources: L-tryptophan, indican, indole-3-acetic acid, indoleacetamide, 5-hydroxyindoleacetic acid, and N-acetyl-L-tryptophan, from the Mann Research Laboratories, 136 Liberty Street, New York 6, N.Y.; indole-3-lactic acid, from Regis Chemical Company, 1219 North Wells Street, Chicago 10, Ill. and 6-hydroxyskatole sulfate as a gift from Dr. E. C. HORNING of the National Institutes of Health, Bethesda, Md. We were unable to obtain a pure sample of indoleacetylglutamine; identification of this compound was made by determination by R_F values in different solvent systems. Indoleacetylglucuronic acid has not yet been synthesized; identification was made on the basis of the presumptive evidence presented in the text of this paper. (See ADDENDUM.)

the correctness of JEPSON's assumption[18] that IAM arises by ammonolysis from indoleacetylglucuronide.

(1) *Tryptophan load test* (test dose of 10 g DL-tryptophan). After tryptophan load, a marked increase in size of the IAM spot was observed.

(2) *HCl hydrolysis*. With HCl hydrolysis of intact urine or urine concentrate, the IAM spot appeared only faintly, if at all, due to the prior hydrolysis of the parent compound, indoleacetylglucuronide, to indoleacetic acid.

(3) *NaOH hydrolysis*. Hydrolysis with NaOH resulted in complete disappearance of IAM in the alkaline ether extract. The only well-defined spot left was urea.

(4) *β-Glucuronidase hydrolysis*. When intact urine or urine concentrate was subjected to β-glucuronidase hydrolysis, IAM appeared only as a faint spot, if at all, in the alkaline ether extract. This again was probably due to the enzymic hydrolysis of indoleacetylglucuronide to indoleacetic acid before the former could be transformed to IAM by ammonolysis during the extraction procedure. This would confirm the correctness of JEPSON's statement[18] about the origin of IAM as discussed above.

By means of recovery experiments, added tryptamine could also be shown to appear in this extract and to give R_F values of 0.71, 0.72. Tryptamine ran close to, but could be distinguished from, IAM. Normal urine extracts, however, contained too little tryptamine for chromatographic detection by this procedure.

B. Alkaline 2-butanone extract (pH 10)

The alkaline 2-butanone extract consistently revealed three well-defined spots with EBR–nitrite spray. These were: (a) a yellow urea spot with R_F = 0.46, 0.45, (b) an orange-brown indican spot with R_F = 0.56, 0.53 and (c) an unknown sky-blue spot with R_F = 0.60, 0.64, which was eventually identified as 6-hydroxyskatole sulfate (6HSK·OS) by SPRINCE *et al.*[13] and independently by NAKAO AND BALL[19]. The compound 6HSK·OS was observed rather infrequently in normal urines (about 30% of urines tested). A fourth spot with R_F = 0.50, 0.88, found occasionally and not always well defined, was identified as indoleacetic acid probably in the form of an ammonium salt resulting from ammoniacal conditions of extraction. Other faint spots often occurred, but these were not characteristic. With *tryptophan load*, this extract generally showed an increase in indican and in the indoleacetate spot, as well as the appearance of a large tryptophan spot (R_F = 0.28, 0.56) and an indolelactic acid spot (R_F = 0.48, 0.82). The latter two, again, were probably in the form of ammonium salts. With *HCl hydrolysis*, all indole spots disappeared, indicating their complete destruction; only the urea spot appeared. After *NaOH hydrolysis*, practically no change was observed. With *β-glucuronidase hydrolysis*, no indoles were lost. Actually, the indoleacetate spot became more intense due to indoleacetic acid liberated from indoleacetylglucuronide by the enzyme.

C. Acid ethyl ether extract (pH 2)

The acid ethyl ether extract consistently showed four well-defined spots with EBR–nitrite spray. These were: (a) a yellow urea spot with R_F = 0.41, 0.43; (b) a 5-hydroxyindoleacetic acid spot with R_F = 0.25, 0.78; (c) an indoleacetylglutamine spot with R_F = 0.31, 0.72; and (d) a "bent-elbow-shaped" spot, which in reality was two spots joined at

the bend. The vertical spot with R_F = 0.39, 0.88 was indoleacetic acid; the fainter horizontal spot with R_F = 0.47, 0.83 was probably a trace mixture of indolelactic acid and N-acetyltryptophan. The last two compounds have very similar R_F values in the solvent systems used. When the extraction pH was varied over a range from 2 to 10, *maximal* spot intensity (extraction) of indoleacetic acid occurred at an extraction pH of 6; at this pH 6 extraction, the horizontal spot did not appear (*i.e.* above trace mixture was not extracted).

(1) *Tryptophan load test.* With tryptophan load, the "bent-elbow" became a triad cluster of spots (indoleacetic acid, R_F = 0.42, 0.90; indolelactic acid, R_F = 0.40, 0.83; and N-acetyltryptophan, R_F = 0.48, 0.84). Under these conditions, the indolelactic acid spot developed above, and somewhat to the right of, the N-acetyltryptophan spot. A small tryptophan spot also appeared at R_F = 0.30, 0.58; although ordinarily, tryptophan did not appear in this extract but rather in the acid 2-butanone extract (pH 2) discussed in the next section (Section D).

(2) *HCl hydrolysis.* After HCl hydrolysis, the 5-hydroxyindoleacetic acid and indoleacetylglutamine spots became lighter indicating partial destruction. The intensity of the indoleacetic acid spot, however, was increased, probably due to hydrolysis of the glucuronide and glutamine conjugates.

(3) *NaOH hydrolysis.* Hydrolysis with NaOH resulted in a marked intensification of all the EBR-reacting spots found in the unhydrolyzed extract. Indolelactic acid became prominent. It is possible that other indoles were liberated by this hydrolysis, but "chromatographic tailing" made identification difficult.

(4) *β-Glucuronidase hydrolysis.* Treatment with β-glucuronidase resulted in a marked increase in the indoleacetic acid spot. Again, this was probably due to the hydrolysis of the glucuronide conjugate.

D. Acid 2-butanone extract (pH 2) (See ADDENDUM).

The acid 2-butanone extract consistently showed well-defined EBR–nitrite spots with the usual R_F values for urea, indican, tryptophan, indoleacetylglutamine, and indoleacetic acid. The intensity of the indoleacetic acid spot in this extract paralleled that of the indoleacetylglucuronic acid spot (discussed below) and probably was derived from it by minor hydrolysis during the extraction procedure.

Indoleacetylglucuronic acid appeared as a blue spot with R_F = 0.68, 0.85 quite often in this extract. It was identified on the basis of the following three observations. Treatment of urine with β-glucuronidase resulted in complete (or almost complete) disappearance of this spot and the indoleacetic acid spot in this extract. Concomitantly, indoleacetic acid was increased in the preceding acid ether extract (see Section C above) after such enzymic hydrolysis. Although detection was difficult due to interfering substances, glucuronic acid could be demonstrated in this spot as follows: From an unstained two-dimensional chromatogram of the acid–butanone extract, an area in R_F region of the glucuronide conjugate was cut out, eluted with water, hydrolyzed with β-glucuronidase, rechromatographed in the same two solvent systems, and sprayed with naphthoresorcinol reagent[20]. By comparison of R_F values and co-chromatography tests with pure sodium glucuronate identification was established. Unhydrolyzed eluate controls treated similarly did not reveal the presence of glucuronic acid. The relationship of indoleacetamide (IAM) to indoleacetylglucuronide was demonstrated by ammonolysis of the unhydrolyzed eluate (*i.e.* adjusting to pH 10

with concentrated NH_4OH and allowing to stand for 30 min at room temperature). When such an ammonolysate was concentrated and subjected to two dimensional chromatography, both IAM and glucuronic acid could be detected. With *HCl hydrolysis*, all indole spots, including indican again disappeared, indicating their complete destruction; only the urea spot remained. After *alkaline hydrolysis*, very little change was noted. If anything, the tryptophan spot was somewhat increased. With *tryptophan load*, an increased intensity of the tryptophan, indoleacetylglutamine, and indoleacetic acid spots was observed. The indoleacetylglucuronic acid spot also was more prominent.

E. Aqueous residues

The aqueous solutions remaining after the butanone extractions under both alkaline and acidic conditions showed very little in the way of well-defined indoles. Only tryptophan was apparent, especially after tryptophan load.

DISCUSSION

The extraction procedure described in this paper offers certain advantages not used heretofore in the isolation of indoles from urine. *First*, the use of sequential solvents of graded polarity under alkaline and acidic conditions permits the fractionation of urinary indoles into multiple extracts with not too much overlapping. *Secondly*, sequential fractionation makes possible the paper chromatographic analysis of urine extracts more highly concentrated than heretofore employed (30 mg urinary creatinine equivalent were used).

Four separate extracts were obtained from normal male urines. Only those indoles which appeared consistently and were chromatographically well-defined soon after spraying with EBR–nitrite are discussed. These are: (1) an alkaline-ether extract containing primarily indoleacetamide; (2) an alkaline-butanone extract containing chiefly indican, 6-hydroxyskatole sulfate (when the latter occurred), and occasionally indoleacetic acid probably as an ammonium salt; (3) an acid-ether extract containing chiefly 5-hydroxyindoleacetic acid, indoleacetylglutamine, indoleacetic acid, and traces of indolelactic acid and acetyltryptophan; and (4) an acid-butanone extract containing chiefly indican, tryptophan, indoleacetylglutamine, indoleacetylglucuronic acid, and a small amount of indoleacetic acid.

Brief comments on the compounds found in these extracts may be in order. Indoleacetamide was shown to be derived chiefly from indoleacetylglucuronic acid by ammonolysis, thereby confirming JEPSON[18]. It could also arise from indolepyruvic acid[18]. Indican appeared in considerable amounts in both alkaline and acid-butanone extracts, but not in the corresponding ether extracts. Indoleacetylglutamine occurred only in the acid extracts, and was generally more prominent (more readily extractable) in acid-butanone than in acid-ether. Indoleacetic acid was extracted chiefly by acid-ether, optimally at pH 6; the small amount in acid-butanone was probably derived from indoleacetylglucuronic acid by minor hydrolysis during the extraction procedure. Indoleacetylglucuronic acid could only be detected in the acid-butanone extract and was extracted optimally at pH 4. Urea was found in all extracts, but in much larger amounts in the butanone extracts. Serotonin could not be detected in any of the normal urine extracts by this procedure.

SUMMARY

1. A method for the extraction and paper chromatographic analysis of urinary indoles is described which utilizes ethyl ether and 2-butanone sequentially under alkaline and acidic conditions.

2. The advantages of this procedure are two-fold: (1) it permits the fractionation of urinary indoles into multiple extracts with not too much overlapping and (2) it makes possible the paper chromatographic analysis of urine extracts more highly concentrated than heretofore employed.

3. The pattern of excretion of urinary indoles in a total of 50 normal male urines (individual or pooled) as demonstrated by this method is presented. The indoles most generally detected in normal male urines were tryptophan, indican, indoleacetic acid, indoleacetylglutamine, indoleacetylglucuronic acid, indoleacetamide, 5-hydroxyindoleacetic acid, 6-hydroxyskatole sulfate (in 30 % of normals), and traces of indolelactic acid, and N-acetyltryptophan.

4. The effect of alkaline, acid, and enzymic (β-glucuronidase) hydrolysis of urine prior to extraction was studied.,

5. The effect of tryptophan load tests is reported.

6. By utilization of this method, the presence of indoleacetylglucuronic acid was demonstrated in the acid-butanone extract and its relationship to indoleacetamide in the alkaline-ether extract was established. (See ADDENDUM.)

REFERENCES

1 A. SJOERDSMA, H. WEISSBACH AND S. UDENFRIEND, *Am. J. Med.*, 20 (1956) 520.
2 H. SPRINCE, *Clin. Chem.*, 7 (1961) 203.
3 J. B. JEPSON, *Lancet*, 2 (1955) 1009.
4 H. H. FLEISCHHACKER AND J. B. LANCASTER, *J. Mental Sci.*, 105 (1959) 777.
5 C. E. DALGLIESH, *J. Clin. Pathol.*, 8 (1955) 73.
6 A. ASATOOR AND C. E. DALGLIESH, *J. Chem. Soc.*, (1956) 2291.
7 D. M. SMITH, R. M. PAUL, E. G. MCGEER AND P. L. MCGEER, *Can. J. Biochem. and Physiol.*, 37 (1959) 1493.
8 D. KEMALI, G. ROMANO AND V. M. BUSCAINO, *Acta Neurol. (Naples)*, 12 (1957) 103.
9 D. KEMALI AND V. M. BUSCAINO, in M. RINKEL AND H. C. B. DENBER, *Chemical Concepts of Psychosis*, Proc. Symp., 2nd Intern. Congr. Psychiatry, Zurich, Switzerland, Sept. 1–7, 1957, McDowell and Obolensky, New York, 1958, p. 219.
10 M. D. ARMSTRONG, K. N. F. SHAW AND P. E. WALL, *J. Biol. Chem.*, 218 (1956) 293.
11 M. D. ARMSTRONG, K. N. F. SHAW, M. J. GORTATOWSKI AND H. SINGER, *J. Biol. Chem.*, 232 (1958) 17.
12 A. HANSON AND F. SERIN, *Lancet*, 2 (1955) 1359.
13 H. SPRINCE, E. HOUSER, D. JAMESON AND F. C. DOHAN, *A. M. A. Arch. Gen. Psychiat.*, 2 (1960) 268.
14 H. SPRINCE, *J. Chromatog.*, 3 (1960) 97.
15 H. SPRINCE, C. M. PARKER, D. JAMESON, J. T. DAWSON, JR., M. KNOWLTON AND F. C. DOHAN, *J. Lab. Clin. Med.*, 57 (1961) 763.
16 H. SPRINCE, *Ann. N.Y. Acad. Sci.*, 96 (1962) 399.
17 J. B. JEPSON, in I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, Interscience, New York, 1960, p. 183.
18 J. B. JEPSON, *Biochem. J.*, 69 (1958) 22 P.
19 A. NAKAO AND M. BALL, *J. Nervous Mental Diseases*, 130 (1960) 417.
20 I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, Interscience, New York, 1960, p. 250.