Chapter 2

Chemical analysis of indolealkylamines and related compounds

By

ARNE HANSON

With 11 figures

Introduction

A close parallel between methodology and understanding has been recognized throughout modern scientific research, and is germane to the topics of indolealkylamines and related compounds. Although 5-hydroxytryptamine was first isolated from biological material by ERSPAMER in 1937, further developments in this field were sparse until the early 1950's, when more advanced analytical techniques were employed. Within the past ten years, isolation, identification, and quantitation of indole compounds has rapidly proliferated, and the metabolic pathways, physiological rôles, and pharmacological effects of these compounds have been increasingly understood. Of crucial importance to this progress has been the application of three analytical techniques: paper chromatography, spectrophotometry, and spectrophotofluorometry.

Indolealkylamines often have marked biological activity. Hence, determination of tissue concentration and excretion may involve measurements in the microgram range. The high sensitivity and narrow specificity of present methods permits examination of indole compounds under physiological circumstances, and recognition of derangements in indole metabolism.

In this review, physical and chemical methods, pertinent to analysis of indolealkylamines and related compounds in biological material will be presented. For practical reasons, emphasis is given to methods which have gained wide usage, or which are judged to have a potentially broad application.

Reviews which cover different aspects of indole analysis have been published by ERSPAMER (1954), DALGLIESH (1958), UDENFRIEND et al. (1958), JEPSON (1960), UDENFRIEND (1962), SANDLER (1963) and UDENFRIEND and WEISSBACH (1963).

A. Isolation and fractionation from biological material

If solid compounds are to be examined no problems of isolation arise; the material is dissolved in a suitable solvent and appropriate identification procedures started (see below). However, biochemical and pharmacological work on indoles mostly concern their occurrence, distribution or fate in animal tissues. Hence, they have to be isolated from tissue before any identification or assay studies can be made. Further, very often the compounds studied occur in tissues in such minute amounts that a concentration procedure must be included in the initial stages of investigation.

In the following section general procedures for the isolation and separation of indoles from biological material will be briefly described. For a thorough discussion of separation techniques, the reader is referred to MORRIS and MORRIS (1963).

I. Extraction procedures

1. Acetone extraction

Acetone has been the classical extraction solvent for indole analysis since it was introduced in 1940 by ERSPAMER for the extraction of 5-HT from tissues (see ERSPAMER 1954, ERSPAMER and BERTACCINI 1962). In the early investigations which led to the isolation of 5-HT as enteramine from mammalian gastrointestinal mucosa and salivary glands of octopods by ERSPAMER and coworkers in 1937 (see ERSPAMER 1954) and as serotonin from serum by RAPPORT and coworkers in 1948 (RAPPORT et al. 1948) acetone was extensively used in the extraction procedures.

Acetone extraction is carried out as follows (ERSPAMER 1955, 1956; ERSPAMER and BERTACCINI 1962):

Tissues. — Tissues are minced with scissors and extracted with 80 per cent aqueous acetone (4 ml of acetone per gram fresh tissues) for 24 hours. After decanting acetone, the tissues are reextracted with the same volume of acetone for another 24 hours. The combined acetone extracts, which can be kept indefinitely at low temperature, are evaporated in vacuo, or, if small volumes are used, by rapid evaporation in a boiling water bath under an air stream. The residue is then dissolved in water or other suitable solvent.

It has been found that 80 per cent acetone assures a complete extraction not only of 5-HT but also of 5-HTP and 5-HIAA (ERSPAMER and BERTACCINI 1962).

It should be stressed that homogenization of the tissues before extraction does not offer any advantages; on the contrary, it may be harmful to 5-HT when tissues rich in monoamine oxidase are analyzed.

Plasma and serum. Samples are treated with 4 volumes of acetone, left standing in a refrigerator overnight and then filtered or centrifuged. The precipitate is washed once with acetone, and the combined acetone extracts evaporated in vacuo and treated as above.

Urine. Urine is concentrated in vacuo, at 40—50°, to a 1/20 of the original volume. 1 volume of 95 per cent ethanol and 4 volumes of acetone are then added with stirring. After standing in a refrigerator for 8—24 hours the precipitate formed is filtered off and the filtrate evaporated in vacuo. The residue is taken up in a mixture of 1 ml water + 3 ml ethanol + 25 ml acetone for every 100 ml of initial urine volume. The solution thus obtained is repeatedly evaporated to dryness, after being successively redissolved in the following solvents: a) 0.5 ml ethanol + 10 ml acetone; b) 0.25 ml ethanol + 20 ml acetone; c) 0.25 ml ethanol + 2—3 ml acetone + 5—10 ml ether. In each instance, the residue which is not soluble is discarded. Finally, the solute is dissolved in a mixture of ethanol and acetone, in which it is stable, in the cold, for months, and used for chromatographic separation of component indoles.

Recovery of most indolic acids is 95 per cent in the final filtrate. If ether insoluble compounds are anticipated in the starting material, ether should be omitted from the final precipitation mixture.

BUMPUS and PAGE (1955) also used acetone for the preparation of extracts of urine containing 5-HT, N-methyl-5-HT and bufotenine.

2. Butanol extraction

Butanol saturated with 0.1 N HCl is a useful solvent for the extraction of most indolic acids from biological material. Extraction may be performed using the original aqueous solution (e.g. urine or tissue homogenates), as the butanol is immiscible with water. However, it is necessary to adjust the solution to 0.1 N with respect to HCl before extraction with the acid butanol.

5-HT can also be extracted by acid butanol if salt is present in the solution (MEAD and FINGER 1961).

However, for the isolation of 5-HT the method of choice is the alkaline butanol extraction procedure introduced by UDENFRIEND et al. (1955b). Although this

method has been somewhat modified in details in successive publications (BOGDANSKI et al. 1956, UDENFRIEND et al. 1958) in brief it is as follows: after acid homogenization of the tissues, followed by adjustment to pH 10, the 5-HT is extracted into n-butanol from a salt-saturated solution buffered with borate to pH 10. Saturation with salt facilitates the extraction of 5-HT into butanol. Washing of the butanol extract with salt-saturated borate buffer removes 5-HTP, TP and normally occurring blank impurities. The addition of heptane to the butanol lowers the polarity, so that 5-HT can be reextracted into dilute acid in which it can be assayed.

This method can, of course, also be used for other indolealkylamines. Bufotenine, which is much more soluble in organic solvents than 5-HT, can be completely extracted into butanol from an alkaline aqueous solution without salt saturation (UDENFRIEND et al. 1958).

A detailed description of the alkaline butanol extraction procedure is given on p. 99.

QUAY (1962) has studied the differential extractions of diverse 5-hydroxy- and 5-methoxyindoles from tissues. By combination of alkaline butanol extraction and p-cymene and ether extractions 5-HT, N-acetyl-5-HT, 5-HTP, 5-HIAA, 5-methoxy IAA, and melatonin can be each selectively extracted.

3. Other solvent extractions

When indolic acids present in biological material (e.g. urine) are studied by paper chromatography they are preferably first isolated by extraction. As most of these acids are soluble in ether or ethyl acetate, a simple extraction with one of these solvents is made. Such an extract eliminates amino compounds and other non-ether soluble compounds which may subsequently interfere with analysis. Moreover, such an extract can be evaporated, yielding a solution ten to twenty times more concentrated and may thus reveal minor components as well as reduce the volume of solution to be applied to the chromatogram.

ARMSTRONG et al. (1958), who studied the indolic acids of human urine, prepared an ethyl acetate extract of the urine preliminary to paper chromatography.

II. Chromatographic procedures

1. Adsorption chromatography

Adsorption chromatography on alumina has been used by several workers for the partial purification and separation of indole derivatives from biological material.

BUMPUS and PAGE (1955) used a 2.3×21 cm alumina column (aluminium oxide, Merck, for chromatography) for the separation of 5-HT, N-methyl-5-HT and bufotenine from human urine. The column was initially prepared in benzene, then washed with benzene diluted with increasing quantities of methanol until a final washing was made with 100 ml of absolute methanol. Urine extracts prepared with acetone (see above) were then added to the column in 5 ml portions of methanol solution and eluted with methanol diluted with increasing amounts of water as follows:

95 ml of absolute methanol,
100 ml of 80% methanol,
100 ml of 60% methanol,
25 ml of 40% methanol,
25 ml of 20% methanol and
50 ml of water.

The eluates were collected in 5 ml fractions. After removal of the methanol at reduced pressure (50°) the indolealkylamines were identified and determined by chromatography and biological assay. Fig. 1 shows the distribution of the three indolealkylamines in such a fractionation.

A similar column was also used by McIsaac and Page (1959) for partial fractionation of metabolites of 5-HT in rats and rabbits.

The same adsorbent has been used by Erspamer and Bertaccini (1962) for a partial purification and separation of 5-HTP metabolites in rat urine. The urine was concentrated in vacuo (50°) to 5 ml and 100 ml of 96 per cent ethanol added. The turbid liquid was transferred to a 3.3×21 cm alumina column (alkaline alumina, Merck, for chromatography) and passed through it. Elution was carried out first with decreasing concentrations of ethanol, then with distilled water, and finally with 10 per cent acetic acid. The eluates were collected in single fractions of 100 ml each, and samples of the different fractions were examined by paper chromatography after suitable concentration.

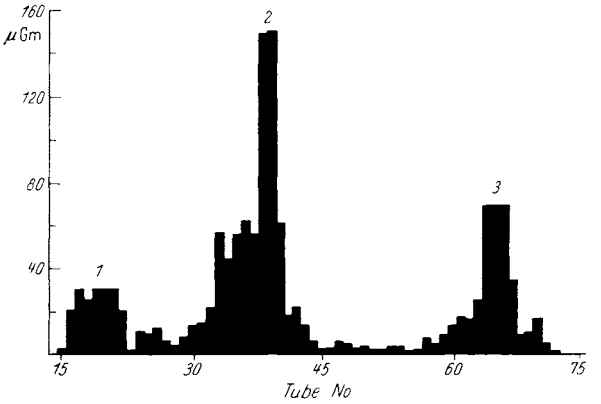


Fig. 1. Aluminium oxide chromatography of bufotenine (peak 1), N-methyl-5-HT (peak 2) and 5-HT (peak 3). From Bumpus and Page (1955).

2. Ion exchange chromatography

Ion exchange chromatography can greatly facilitate both isolation and fractionation of indoles occurring in biological material at extremely low concentrations.

Generally, indolealkylamines are taken up by a weak acid exchanger (e.g. Amberlite IRC-50 or Zeo-Karb 226) at pH 6—8, under which conditions the amines exist as cations. The amines are eluted either with acid, which suppresses the ionization of the exchanger, or with alkali, which suppresses the ionization of the amine.

In Table 1 are given some of the ion exchange resins which have been used for the isolation of indolealkylamines.

Table 1. *Some ion exchange resins used for the separation of indolealkylamines and related compounds*

Exchange resin	Indoles studied	Reference
Amberlite IRC-50 . .	5-HT	Dengler (1959), Oates (1961)
Amberlite XE-97 . .	5-HT	Gluckman and Abrams (1959)
Amberlite CG-50 . . .	5-HT and T	Davis and Awapara (1960), Cooper and Melcer (1962), McIlwain and Rodnight (1962)
Zeo-Karb 226	Indolealkylamines	Rodnight (1956), Blau (1961)
Dowex 1	5-HT and 5-HTP	Schanberg (1962)
Dowex 50	Phenolic amines (incl. 5-HT)	Kakimoto and Armstrong (1962)
Permutit	5-HT	Lovenberg et al. (1962)

RODNIGHT (1956) used columns containing 4 g of Zeo-Karb 226 (H form, 90—120 mesh). After repeated regeneration with 1 N H_2SO_4 and 1 N NaOH and washing with water, the resin was buffered with 0.5 M Na_2HPO_4-HCl buffer pH 6. A 75—120 ml sample of urine, adjusted to pH 6 and diluted to 250 ml with water was then run through the column. The resin was then washed with 125 ml de-gassed water, followed by 125 ml de-gassed 50 per cent ethanol. The indolealkylamines were then eluted with 350 ml of 0.02 N acetic acid in 75 per cent ethanol.

BLAU (1961) has used the same ion exchange resin for the separation of amines (including indolealkylamines) from biological material. However, here the resin was buffered at pH 7.3 and the amines were eluted with buffer pH 5.

MCILWAIN and RODNIGHT (1962) have described a similar method for the isolation of 5-HT and T from urine but use Amberlite CG-50 buffered at pH 6 with 0.5 M Na_2HPO_4—HCl. Columns (1 cm diameter) are loaded with 2 g (dry weight) of the resin which is regenerated with acid and buffered as described by RODNIGHT (1956) for Zeo-Karb 226, except that the alkaline washing is omitted. The diluted urine is allowed to run through the column at about 100 ml/hr. and the effluents discarded. The column is washed with 40 ml of water. The amines are eluted with 30 ml of 2 N H_2SO_4 after running through and discarding 4 ml of 2 N H_2SO_4. The flow rates for both stages of elution should not exceed 1 ml/min. The eluates are collected in centrifuge bottles and are made alkaline (pH 10—11) by gradual addition of 6 g of anhydrous Na_2CO_3. 60 mg of ascorbic acid and 60 ml of n-butanol-benzene (1:1) are added and the bottles are shaken and centrifuged. Of the upper phases 50 ml are transferred to bottles containing 30 ml of heptane and 5 ml of 2 N formic acid. The mixtures are shaken for a few minutes and centrifuged. The whole of the aqueous layer is removed and taken to dryness by freeze-drying or in vacuo evaporation. The residues are then dissolved in 80 per cent methanol and used for paper chromatography.

Another carboxylic acid resin suitable for the isolation of 5-HT and other indolealkylamines is Amberlite IRC-50. This resin was used by OATES (1961) for the determination of 5-HT in urine. A description of his method is given on p. 102.

DENGLER (1959) also used Amberlite IRC-50 for the isolation and separation of 5-HT and 5-HIAA from carcinoid tumour tissues. The tumour tissues are homogenized with 5 per cent perchloric acid. The homogenate centrifuged and the supernatant adjusted to pH 6 with KOH. The precipitate of perchlorate is removed by filtration and the filtrate run through an Amberlite IRC-50 column equilibrated with 1 M acetate buffer pH 6.5. 5-HIAA passes through the column and is determined in the effluent. 5-HT is eluted with dilute H_2SO_4.

The carboxylic acid resins may be most useful in the separation of substrate and reaction product in studies involving the 5-HTP decarboxylase. 5-HTP and 5-HT present in the incubation mixtures can be easily separated on resins such as Amberlite CG-50 (DAVIS and AWAPARA 1960).

Indolealkylamines like 5-HT and T can also be separated from their corresponding amino acids by cation exchange absorption on Permutit. LOVENBERG et al. (1962) in their studies on the L-amino acid decarboxylase applied a Permutis method originally described by DIETRICH (1953). Aliquots of the incubate (pH 9) were diluted to 5 ml with water. After centrifugation the supernatant solution was passed through a 0.5×3 cm Permutit column. The column was washed with 20 ml of boiling water to remove the amino acid, and the amine was then eluted with 3 ml of 20 per cent NaCl. The recovery of added amine in the salt eluate ranged between 90 to 100 per cent.

In the concentration and purification of phenolic amines of human urine, KAKIMOTO and ARMSTRONG (1962) used the strong acid cation exchanger Dowex 50.

3. Paper chromatography

Paper chromatography, which will be discussed here primarily as a method of identification, may, however, be most useful as a method for isolation and separation of small amounts of indole compounds.

Chromatograms employed primarily for identification may be contrasted with those used to achieve isolation of a given substance. In the former, minute volumes are applied as a small, discreet spot; in the latter, thick papers, heavily overloaded with starting material are used.

For isolation one-way chromatograms are frequently used and the solution is applied as a large streak parallel to the origin line. When thick papers, such as Whatman 31 or 3 MM, are used, up to 0.2 ml of solution may be applied and after drying the paper, application of the same volume may be repeated several times prior to the chromatographic run. Thus, relatively large volumes may be conveniently chromatographed. The papers are then chromatographed in the usual way and the components separate in horizontal bands. After drying, the indole compounds are located using suitable reagents. Physical methods (e.g. ultraviolet fluorescence) are preferable but a guide-strip (1—2 cm wide) may also be cut from one side of the paper, in the direction of the solvent flow, and treated with the location reagent to find the position of the compound. This strip is then realigned with the untreated paper. The material is eluted from the paper by cutting it into small pieces and extracting with solvent.

With the above technique it may be possible to isolate indole compounds in amounts up to the milligram level. For further technical details, the reader is referred to BLOCK et al. (1958), HAIS and MACEK (1958), LINSKENS (1959) and SMITH (1960).

Larger amounts of material can be isolated by use of column procedures. It is often found that columns of cellulose powders will allow separations similar to those obtained on paper if the same solvents are employed. A preliminary paper chromatographic analysis may thus be useful to guide a later large scale separation on a cellulose powder column.

ERSPAMER and NOBILI (1962a) have isolated metabolites of 4-HTP on a cellulose powder column. Eluates from an alumina column (see p. 69) were concentrated and chromatographed on a column of Whatman 1 cellulose powder using the ascending technique and a solvent of n-butanol-acetic acid-water (4:1:5). The column was prepared in a glass tube formed by two adherent hemi-cylinders which could be opened longitudinally. The tube was positioned upright, exactly as if it were a paper sheet and placed into a chromatographic cabinet with the solvent at the bottom. After a convenient run, the tube was taken out. To locate the desired compounds, the column was opened longitudinally, paper strips were pressed gently on to the column surface, and then removed and sprayed with NNCD reagent (see p. 87). From the location of the coloured spots on the paper, the column segment containing the desired material could be accurately identified and subsequently eluted. This technique exactly parallels the "guide-strip" method as described above.

4. Molecular sieving (gel filtration)

Cross-linked dextran gels have recently been developed for molecular sieving. Solutes vary greatly in their ability to penetrate the pores of such gels and the extent of their penetration reflects primarily the molecular size of the solute.

Different grades of dextran gels (designated Sephadex G-25, G-50, G-75, G-100 and G-200) are commercially available. Sephadex G-25 will completely exclude neutral compounds with a molecular weights greater than 3500—4500. The process of separating molecules of different size by passage through a column of a dextran gel is termed *gel filtration* (for technical details, see FLODIN and PORATH 1961, BOARDMAN 1962, MORRIS and MORRIS 1963). The chromatographic behaviour of small molecules on Sephadex G-25 has been thoroughly investigated by GELOTTE (1960) and his paper should be consulated for details. Substances of similar size may also show a difference in migration rates, because dextran gels are not completely devoid of sorption and ion exchange effects.

Table 2. *Separation of indole compounds on Sephadex G-25.* (SCHLOSSBERGER et al. 1963)

Compound	Fraction number
Urea	16—26
Citrulline	18—24
Indican	26—30
Tryptophan	28—39
Tryptamine	34—50
Indoleacetic acid	40—45
5-Hydroxytryptophan	39—48
5-Hydroxytryptamine	46—66
5-Hydroxyindoleacetic acid	42—60
5,6-Dihydroxytryptophan	36—46
5,6-Dihydroxytryptamine	44—52
5,6-Dihydroxyindoleacetic acid	44—50

SCHLOSSBERGER et al. (1963) have used gel filtration with Sephadex G-25 for the separation of indole derivatives from urine. A 10 ml sample of the urine is first concentrated to 2 ml by evaporation. An 0.8 ml aliquot of the centrifuged concentrate is applied to a 1.4×20 cm Sephadex G-25 column which has been equilibrated with 0.5 per cent ascorbic acid solution to prevent oxidation of the unstable 5,6-dihydroxyindoles. The elution of the indoles is also made with 0.5 per cent ascorbic acid solution and the eluates collected in 1 ml fractions. The indoles are then identified in the eluates by means of thin layer chromatography (adsorbent: cellulose powder; solvent: butanol-acetic acid-water, 12:3:5; spray reagent: p-dimethylaminocinnam aldehyde).

Table 2 shows the distribution of a number of indole derivatives on Sephadex G-25.

III. Paper electrophoresis

Paper electrophoresis will be discussed here mainly as an identification method. However, it may be used as an isolation and separation procedure in the same way as described above for paper chromatography. Most paper electrophoretic techniques lend themselves to the separation of quantities up to a few hundred milligrams, by employing either a stack of filter paper or a single thick paper strip. For further details, see LEDERER (1955).

B. Identification

I. Ultraviolet absorbancy

The absorption spectra of 5-hydroxyindoles in neutral and acid solution show a maximum in the ultraviolet at 275 mμ with a subsidiary peak at 295 mμ. In alkali, the subsidiary peak is shifted to 325 mμ (Fig. 2). The molecular extinction of 5-HT in acid at 275 mμ is 5.3×10^3 (UDENFRIEND et al. 1958).

The introduction of a second hydroxy group at position 6 of the indole ring results in a reversal of the magnitude of the peaks described above. For 5,6-dihydroxytryptophan in neutral solution, the larger peak is at 295 mμ, while the subsidiary peak is at 265—270 mμ (SCHLOSSBERGER and KUCH 1963, see Fig. 3).

Simple indoles which are not substituted in the ring show an absorption maximum at 280 mμ with a small subsidiary peak at 288 mμ in both acid and alkaline solutions (Fig. 4). This lack of acid-base shift of ultraviolet absorption maxima seen in simple indoles is also found in compounds which have their ring hydroxyls blocked by methylation (e.g. melatonin and 5-methoxy-IAA), see Fig. 5.

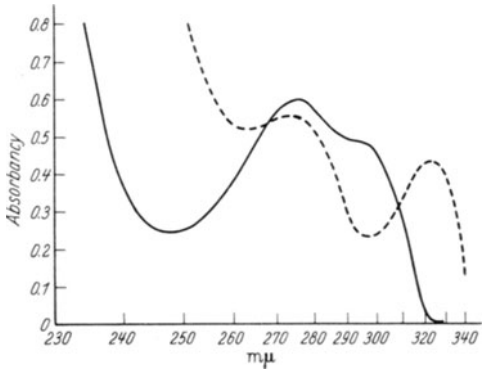


Fig. 2

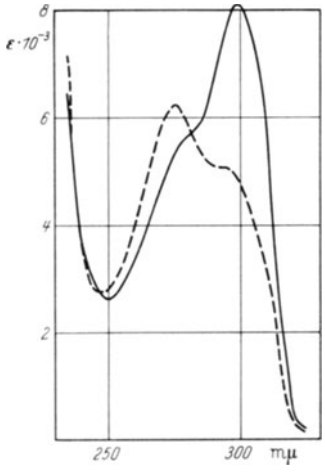


Fig. 3

Fig. 2. Spectral absorbancy curves of 5-HT (0.1 μM per ml) in 0.1 N HCl (———) and in 0.1 N NaOH (— — — —)

Fig. 3. Ultraviolet absorption spectra of DL-5-HTP (— — — —) and DL-5,6-dihydroxy-TP (———) in water (SCHLOSSBERGER and KUCH, 1963)

Measurement of absorption in acid at 275—280 mμ is the simplest procedure for the determination of indoles. However, the low sensitivity and lack of specificity make it inapplicable to studies of endogenous indoles in biological samples.

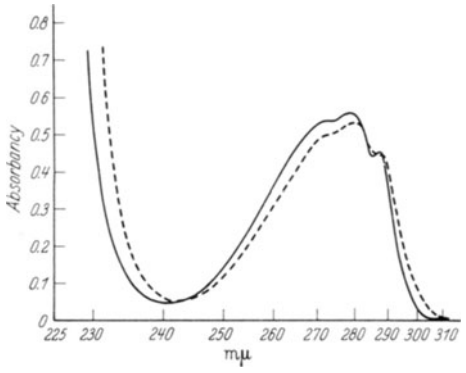


Fig. 4

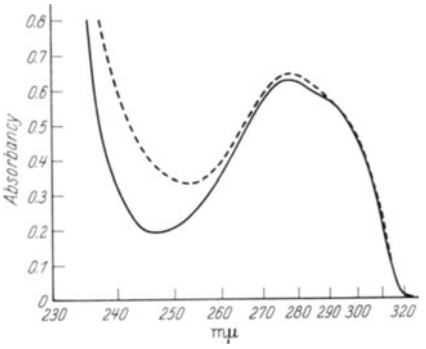


Fig. 5

Fig. 4. Spectral absorbancy curves of T (0.1 μM per ml) in 0.1 N HCl (———) and in 0.1 N NaOH (— — — —)

Fig. 5. Spectral absorbancy curves of melatonin (0.1 μM per ml) in 0.1 N HCl (———) and in 0.1 N NaOH (— — — —)

It has been successfully applied, however, to the measurement of enzymatic formation and destruction of 5-HT in tissue preparations (UDENFRIEND et al. 1955b, DAVIS and AWAPARA 1960). The amount of 5-HT in such experiments is large and the blank is minimal, because a comparatively small amount of tissue is taken through the extraction procedure.

II. Fluorescence

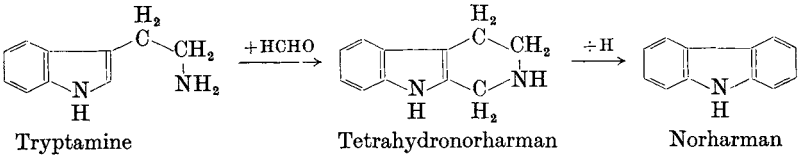
Many of the biologically occurring indoles exhibit a very intense fluorescence which make them readily detectable in minute amounts in extracts and on chromatograms.

Indoles which are not substituted in the ring (e.g. TP, T and IAA) fluoresce in weak acid or neutral solutions at 350 mμ, when activated at 275 mμ, and 5-hydroxyindoles (5-HTP, 5-HT and 5-HIAA) emit a maximum fluorescence at 350 mμ, when activated at 295 mμ (BOWMAN et al. 1955).

In strong acid (3 N HCl) 5-hydroxy- and 5-methoxyindoles fluoresce at 550 mμ and this fluorescence is also maximally activated at 295 mμ. This shift of fluorescence, from the ultraviolet to the visible, with increasing acidity, is completely reversible and is not accompanied by a noticable change in the absorption spectrum. No corresponding shift in fluorescence is observed with the simple indoles. In fact, these compounds loose their fluorescence in 3 N HCl. The fluorescence shift in strong acid is a function of the phenolic group and distinguishes 5-hydroxy- and 5-methoxy-indoles from other indoles (UDENFRIEND et al. 1955a, QUAY 1963).

Fluorescence assay permits better discrimination among indole compounds than do spectrophotometric and colorimetric methods. All 5-hydroxyindoles have exactly the same colour with several reagents, e.g. 1-nitroso-2-naphtol. In neutral or weak acid solutions they are all activated to fluoresce by ultraviolet light of the same wavelength (295 mμ) and emit the same fluorescence at 350 mμ. However, each compound has its own optimal pH for maximal fluorescence. Thus, it becomes possible to distinguish between various 5-hydroxyindole derivatives by selecting the proper pH in which to make the fluorescence measurements.

In addition to their native fluorescence, several indole compounds give highly fluorescent reaction products when treated with certain reagents. Indoleethylamine derivatives (e.g. T) condense with formaldehyde to the nonfluorescent tetrahydronorharman which on dehydrogenation with hydrogen peroxide forms the highly fluorescent norharman, 3-carboline (HESS and UDENFRIEND 1959).



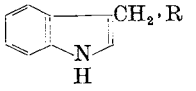
Tryptamine — Tetrahydronorharman — Norharman

This reaction is used in a very sensitive method for the determination of T in tissues (see p. 95).

Another reaction also based on the formation of a carboline derivative is that described by JEPSON and STEVENS (1953) for the detection of certain tryptamines on paper chromatograms. When 5-HT and other primary indole-alkylamines are treated on paper chromatograms with ninhydrin in acetic acid they yield 3,4-dihydro-3-carbolines (JEPSON 1960). These derivatives show an intense greenish-blue fluorescence in ultraviolet light, detectable at levels as low as 0.01 μg per cm^2 paper. However, here the fluorescence only occurs when the 3,4-dihydro-3-carbolines are adsorbed on paper (see p. 88).

PROCHÁZKA (1953) has developed a fluorescence reaction for the detection of indole compounds on paper chromatograms . When the latter are sprayed with a mixture of formaldehyde and hydrochloric acid most indoles substituted at position 3 show orange to brown colours with characteristic yellow or orange

fluorescence (see p. 88). The substituted group must be separated from the ring system by a methylene group.

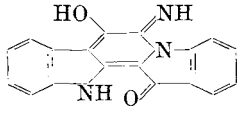


However, derivatives where R is a OH-, indolyl- or dimethylamine group only show red to violet colours.

A similar reaction has been described by SHEPHERD et al. (1953) who use formaldehyde and potassium dichromate. When treated on chromatograms with this reagent, 5-HT, bufotenin and bufotenidin produce a golden-yellow fluorescence in amounts as small as 0.2 μg (see p. 89).

Although histochemical methods will not be treated in this review, mention must be made here of the highly sensitive fluorescence methods for histochemical detection of catecholamines and 5-HT at the cellular level, as developed by FALCK and coworkers (see FALCK 1962). The technique used in these methods is briefly as follows: Pieces of the tissues to be studied are frozen in propane cooled by liquid nitrogen and dried in vacuo at —35°. After thorough drying the pieces are treated with formaldehyde gas at +80° in a closed glass vessel containing paraformaldehyde. During this treatment 5-HT condenses with formaldehyde yielding a product with intense yellow fluorescence, which is not extracted with hot paraffin or xylene. The preparations are then inbedded in vacuo with paraffin and sectioned. The sections (8—10 μ) are placed on nonfluorescent slides and mounted. The details of the technique and the equipment used for fluorescence microscopy are described by FALCK (1962). Results obtained with this technique have shown that it has a sensitivity much greater than that of hitherto available histochemical methods for 5-HT. This is well illustrated by the following findings: The 5-HT in normal rat mast cells, which has hitherto been beyond the reach of histochemical detection, gives a very strong fluorescence, and single granules from disrupted cells are readily seen. Further, the 5-HT in blood platelets shows a distinct fluorescence when the content exceeds about 100 μg per ml.

TP and T form the highly fluorescent tryptochrome when oxidized in acetic acid with iodate (FEARON and BOGGUST 1950):



Tryptochrome

As a fluorescent test for TP and T the sensitivity of the tryptochrome reaction is about 1 in 10^6. EGELHAAF (1957) has reported a very sensitive chromatographic method for TP in which the tryptochrome is measured fluorometrically.

HOLMSTEDT and LUNDGREN (1964) have found that solutions of psilocin (N,N-dimethyl-4-hydroxytryptamine) turn yellow at pH 12 and fluoresce at 500 mμ, when activated at 370 mμ. Psilocybin (N,N-dimethyl-4-phosphoryltryptamine) does not show any fluorescence at this pH.

The development of spectrophotofluorometers capable of delivering high-intensity monochromatic activation at all wave lengths from 240 to 800 mμ, and concomitant availability of automatic spectral analysis of resulting fluorescence throughout the same range have brought extreme sensitivity into fluorometric methodology (UDENFRIEND 1962). In many cases these methods, which allow the detection of indoles in amounts far below the microgram level, offer distinct advantages over other physical and chemical methods.

Table 3. *Fluorescence characteristics of some biologically important indoles*

Compound	Medium	Activation mμ	Fluorescence mμ	Reference
Tryptophan derivatives:				
Tryptophan	pH 2—11	275	360	1,3
Tryptophan	as norharman	365	440	5
Tryptamine	pH 2—11	275	360	1,3
Tryptamine	as norharman	365	440	5
Indoleacetic acid	pH 2—11	275	360	1,3
4-Hydroxytryptophan derivatives:				
4-Hydroxytryptamine	pH 7	none	none	13
4-Hydroxytryptamine	3 N HCl	none	none	13
N,N-Dimethyl-4-hydroxytryptamine (psilocin)	pH 12	370	500	14
N,N-Dimethyl-4-phosphoryltryptamine (psilocybin)	pH 12	none	none	14
5-Hydroxytryptophan derivatives:				
5-Hydroxytryptophan	pH 2—11	295	330	1,3
5-Hydroxytryptophan	3 N HCl	295	550	2
5-Hydroxytryptamine	pH 2—11	295	330	1,3
5-Hydroxytryptamine	3 N HCl	295	550	2
N,N-Dimethyl-5-hydroxytryptamine (bufotenine)	3 N HCl	295	550	4
N-Acetyl-5-hydroxytryptamine	3 N HCl	295	550	4
5-Methoxytryptamine	3 N HCl	295	550	4
N-Acetyl-5-methoxytryptamine (melatonin)	pH 7	290	360	6
N-Acetyl-5-methoxytryptamine (melatonin)		304	333, 338	12
N-Acetyl-5-methoxytryptamine (melatonin)	3 N HCl	300	540	4,6
5-Hydroxyindoleacetic acid	pH 2—11	295	330	1,3
5-Hydroxyindoleacetic acid	3 N HCl	295	550	2
5-Methoxyindoleacetic acid	3 N HCl	295	550	4
5-Methoxyindoleacetic acid		304	338	11
6-Hydroxytryptophan derivatives:				
6-Hydroxytryptamine	pH 7	290	365	13
6-Hydroxytryptamine	3 N HCl	none	none	13
6-Hydroxyindoleacetic acid	pH 7	290	365	13
7-Hydroxytryptophan derivatives:				
7-Hydroxytryptamine	pH 7	none	none	13
7-Hydroxytryptamine	3 N HCl	none	none	13
7-Hydroxyindoleacetic acid	pH 7	none	none	13
Other indole compounds:				
Indole	pH 2—11	275	360	1,3
5-Hydroxyindole	pH 1	290	355	7
Lysergic acid diethylamide (LSD)	pH 7	325	465	8,9
Lysergic acid diethylamide (LSD)	pH 9	315	440	1
2-Brom-lysergic acid diethylamide (Brom-LSD)	pH 1	315	460	7,8
Harmine	pH 1	330, 365	400	7,8
Reserpine	pH 1	300	375	7,8
Reserpine	selenious acid oxid.	365	420	10

References to Table 3. [1] BOWMAN et al. (1955). — [2] UDENFRIEND et al. (1955). — [3] SPRINCE et al. (1957). — [4] QUAY (1963). — [5] HESS and UDENFRIEND (1959). — [6] AXELROD and WEISSBACH (1961). — [7] Data sheet to Aminco bulletin, No. 2278. — [8] UDENFRIEND et al. (1957). — [9] AXELROD et al. (1957). — [10] HESS et al (1956). — [11] LERNER et al. (1959a). — [12] LERNER et al. (1959b). — [13] JEPSON et al. (1962). — [14] HOLMSTEDT and LUNDGREN (1964).

Fluorescence characteristics of biologically important indoles and some other related compounds are given in Table 3. For the instrumentation and general principles of spectrophotofluorometric analysis the reader is referred to the monograph of UDENFRIEND (1962).

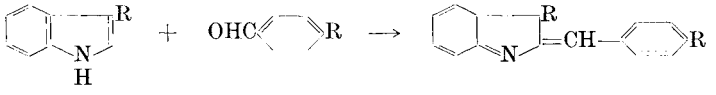
III. Colour reactions

The literature pertinent to colour reactions of indole compounds is voluminous, and will not be extensively reviewed here. Only methods which confer particular advantage in terms of either specificity or sensitivity will be described.

1. Aldehyde reactions

Most indoles condense with aromatic aldehydes to form coloured derivatives. The oldest reaction of this type — introduced by Ehrlich already in 1901 — is that involving p-dimethylaminobenzaldehyde.

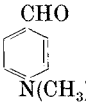
The aldehydes react with the indoles at the free 2- or 3-position. As most biologically important indoles are already substituted at their 3-position the condensation consequently takes place at the 2-position, i.e.



Indole Derivative Aromatic Aldehyde Coloured Reaction Product

Other aldehydes often used for the detection and quantitative determination of biologically important indole compounds are p-dimethylaminocinnamaldehyde and cinnamaldehyde.

a) p-Dimethylaminobenzaldehyde

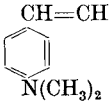


p-Dimethylaminobenzaldehyde in acid solution — often called EHRLICH's reagent — is the most useful of the general tests for indoles and has been widely used for both the detection and quantitation of biologically important indole compounds. It reacts with a wide range of indoles, and shows significant variations in colour development which are useful in the identification of a specific compound concerned.

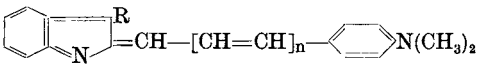
Simple compounds like TP and its derivatives (e.g. T and IAA) show some shade of purple. Substitution of OH- or OR- at position 5 shifts the colour towards blue. Substitution at position 6 changes it to blue-green.

The p-dimethylaminobenzaldehyde reaction is not absolutely specific for the indole nucleus, but a blue colour developing rapidly in the cold is a very good indication of an indole. Purple colours are, however, more dubious and cannot *per se* be attributed to indoles as certain non-indoles give similar colours. Among such naturally occurring compounds should be mentioned simple pyrroles like porphobilinogen, pyrrol-2-carboxylic acid (from mucoproteins) and 2-methyl-pyrrol (from hexosamine).

b) p-Dimethylaminocinnamaldehyde

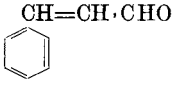


STRELL and KALOJANOFF (1954) suggested the use of vinyl homologues of p-dimethylaminobenzaldehyde for the detection of pyrroles. These compounds also react with indoles to form intensively coloured products.



p-Dimethylaminocinnamaldehyde was used by HARLEY-MASON and ARCHER (1958) for the detection of indoles and reported to be preferable to p-dimethylaminobenzaldehyde in paper chromatography. It is, in fact, in most cases about ten times more sensitive than the p-dimethylaminobenzaldehyde reagent. Its selectivity is, however, low and the background colour may be troublesome (JEPSON 1960). Certain indoles which do not react with p-dimethylaminobenzaldehyde (e.g. β:β-dimethyltryptamine) can, however, be detected with this reagent.

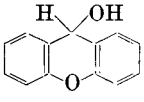
c) Cinnamaldehyde



Cinnamaldehyde reacts in acid solution with indoles in the same way as the other aromatic aldehydes already described. However, the reaction products formed are differently coloured. While the products formed with p-dimethylaminobenzaldehyde and its vinylogue are mostly purple or blue, those formed with cinnamaldehyde are red to brown.

The cinnamaldehyde reaction has been used most frequently for the detection of indoles in paper chromatography and paper electrophoresis (MÜLLER 1953).

2. Xanthydrol reaction



Indole compounds react with xanthydrol in acid solution to form highly coloured acid-stable products (DICKMAN and WESCOTT 1954; DICKMAN and CROCKETT 1956). In the presence of excess xanthydrol the formation of xanthydrylindoles is directly related to the concentration of indole compound in the solution.

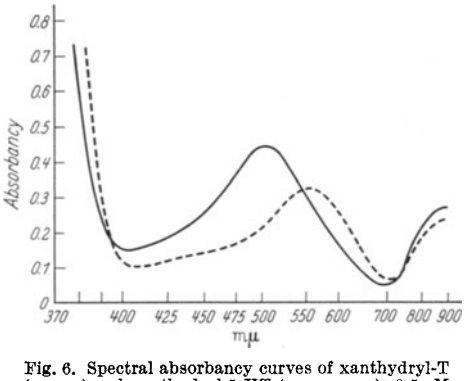


Fig. 6. Spectral absorbancy curves of xanthydryl-T (———) and xanthydryl-5-HT (— — — —). 0.5 μM of the compound in 5 ml of water was treated with 1 mg of xanthydrol in 5 ml of 6N HCl for 15 minutes at 100° and the solution extracted twice with benzene before absorbancy was measured

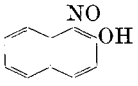
The xanthydryls formed with simple indoles such as TP, T and IAA show an absorption peak at 510 mμ. A hydroxy group in position 5 shifts this peak to 557 mμ (Fig. 6).

Briefly, a sample to be analyzed should contain 0.1 to 0.5 μM of the indole compound in a maximal volume of 5 ml; it is added to a mixture of 1 ml of xanthydrol (1 mg per ml) in concentrated HCl and 4 ml of concentrated HCl. This solution is then diluted to 10 ml with H_2O and the tube heated in a boiling water bath for 15 minutes. After cooling it is extracted three times with wet benzene, and the excess benzene is evaporated from the aqueous phase with a stream of air. The solution is finally diluted to volume with 6 N HCl. The

content of indole compound can be calculated from the absorbancy of the solution at 510 mμ form a calibration curve.

Detailed procedures for the quantitative determination of TP and 5-HT have been reported by DICKMAN and CROCKETT (1956).

3. 1-Nitroso-2-naphtol reaction



This reagent, which was originally introduced by GERNGROSS et al. (1933) for the detection of p-substituted phenols (tyrosin and tyramine) reacts almost exclusively with 5-hydroxyindoles to form coloured derivatives under certain conditions (UDENFRIEND et al. 1955b).

In dilute HCl or H_2SO_4, containing traces of nitrite, 5-hydroxyindoles react to give a violet-purple chromophore, the structure of which is as yet unknown. Tyramine and tyrosin react with 1-nitroso-2-naphtol only in the presence of strong nitric acid. TP, T, 7-HTP and 7-HT do not react at all, and of twenty phenolic compounds reported by UDENFRIEND et al. (1955b) only p-hydroxyacetanilide gave a positive colour reaction. However, recently other compounds — mostly urinary metabolites of ingested drugs — have been found to give a colour similar to that given by 5-hydroxyindoles (see p. 105).

Colorimetric methods based on the 1-nitroso-2-naphtol reaction have been used for analyses of the following indoles: 5-HIAA acid in urine; 5-HT in carcinoid tumours; enzymatic experiments involving 5-HTP and 5-HT. These methods have about the same sensitivity as the ultraviolet absorption technique, but are preferable when tissue extracts contain large amounts of ultraviolet-absorbing material.

The following procedure can be employed for the detection and determination of all the 5-hydroxyindoles after they have been separately isolated (UDENFRIEND and WEISSBACH 1963).

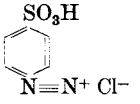
To 2 ml of an acid extract containing 0.03 to 0.8 uM of 5-hydroxyindole in a glass-stoppered shaking tube are added 1 ml of 1-nitroso-2-naphtol reagent (0.1% in 95% ethanol). After thorough mixing, 1 ml of nitrous acid reagent (freshly prepared mixture of 5 ml 2 N H_2SO_4 or HCl and 0.2 ml 2.5% $NaNO_2$) is added. The tube is stoppered, shaken again, and allowed to stand at room temperature for 10 minutes. 10 ml of ethylene dichloride are then added, and the tube is shaken for a sufficient time to extract all excess 1-nitroso-2-naphtol. After low speed centrifugation the aqueous layer is transferred to a cuvette, and the optical density of the stable purple chromophore measured at 540 mμ in a spectrophometer.

4. Diazo reactions

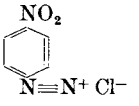
Phenolic compounds, including 4-, 5- and 6-hydroxyindoles, react with diazonium salts (diazo coupling) to give azo dyes which are strongly coloured. These reactions are very sensitive and often allow the detection of hydroxyindoles below the microgram level. In non-hydroxylated, and in such compounds where the phenolic group is blocked by ether or ester formation little colour occurs with simple diazonium salts. However, certain complex diazonium salts give colours with indole acids and indolealkylamines and these salts may be useful for identification.

The diazonium compounds which give the most satisfactory results in indole analysis are the following:

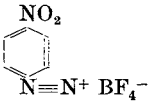
a) Benzenesulphonic acid diazonium chloride, diazotized sulphanilic acid, VAN DEN BERGH'S or PAULY'S reagent



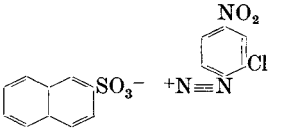
b) p-Nitrobenzenediazonium chloride, diazotized p-nitroaniline



c) p-Nitrobenzenediazonium tetrafluoroborate



d) 2-Chloro-4-nitrobenzenediazonium naphtalene-2-sulphonate, NNCD reagent



Unfortunately, most solid diazonium salts are explosive. Even small quantities of these compounds may explode violently on mechanical shock and cause accidents; hence they are rarely prepared in the dry state. The first two of the diazonium salts mentioned above are such compounds and are therefore mostly prepared in aquoeus solutions. These solutions are not stable and must be prepared fresh before use.

Diazonium salts form complexes with many metallic salts, of which one of the most important is the fluoroborate, $(Ar \cdot N_2)^+BF_4^-$. These complex salts can be prepared in the dry state and handled with relative safety. They are also stable in solution and hence offer a means of stabilizing a diazonium salt solution. One such compound is p-nitrobenzediazonium tetrafluoroborate. Aqueous solutions of this compound are stable even at room temperature. The solid reagent is easily prepared (CHERONIS and ENTRIKIN 1957) and is also commercially available[1]. It is a very effective reagent for the detection of certain indoles and may be useful in the differentiation of hydroxylated and non-hydroxylated compounds (see p. 87).

Another stabilized and safe diazonium compound is 2-chloro-4-nitrobenzenediazonium naphtalene-2-sulphonate (NNCD reagent), which is also commercially available in solid form[2]. This compound was introduced by HEINRICH and SCHULER (1948) for the determination of dihydroxyphenols and has been extensively used for the detection of indoles in paper chromatography (ERSPAMER and BORETTI 1951, ERSPAMER 1954, 1955; see p. 87). Not only phenolic indoles, but also IAA and T react with this reagent.

With phenolic compounds, diazo coupling is best performed in slightly alkaline solution (alkaline coupling). However, with certain diazonium salts, a coupling occurs also in acid solution (acid coupling). The extent of coupling and the resulting colour vary considerably — as a function of pH. When diazo reactions are used for analytical purposes both acid and alkaline coupling should be used, as the colours obtained very often show marked differences which may be characteristic of a given compound.

[1] Eastman Organic Chemicals, Rochester, U.S.A.
[2] Hopkin & Williams Ltd. Chadwell Heath, Essex, England.

In indole analysis the diazo reactions are almost exclusively used after separation by paper chromatography. Chromatograms are first sprayed with the acid diazonium salt, and examined for the appearance of colour. Then, an alkaline pH is obtained by spraying with sodium carbonate, and again the appearance of colours is noted (see p. 87).

6-Hydroxy- and 6-alkoxyindoles with a free 2-position react immediately with diazotized sulphanic acid or p-nitroaniline in strong acid to give brillant red colours (JEPSON et al. 1962). Indoles hydroxylated at positions 4, 5 or 7 only give slowly forming brownish-purple or yellow colours. The sensitivity of the reaction with diazotized sulphanilic acid in strong acid is several times as great for 6-hydroxyindoles as for 7-hydroxyindoles. The absorption spectra of the coloured products from 6-HT and 6-HIAA have peaks at 525 mμ and 510 mμ, respectively. The colour obtained with 7-HT and 7-HIAA show a broader maximum at 555 mμ, with an absorbancy only 0.3 that of 6-HT at their respective maxima. JEPSON et al. (1962) have used this reaction with diazotized sulphanilic acid for the quantitative determination of 6-hydroxyindoles.

IV. Countercurrent distribution

Countercurrent distribution is a separation process, based directly on the principle that a pure solute, when shaken with two immiscible solvents in which it is soluble, will distribute itself between the two liquid layers according to a certain characteristic preference. The preference at a given concentration level and temperature is a constant determined by the Nernst distribution law.

Countercurrent distribution is used mostly for the separation of organic substances, and offers certain advantages in the isolation of biological products: it avoids extremes of temperature; it is applicable both on a preparative scale and at micro levels. Several excellent reviews of countercurrent distribution are available, e.g. HECKER (1955), CRAIG and CRAIG (1956), TITUS (1960) and CRAIG (1961).

The pattern of distribution of a substance in the countercurrent distribution system can be predicted from its partition coefficient by very simple mathematical procedures which greatly facilitate the analytical uses of this process. One common application is to measure small amounts of impurities by means of the discrepancies between observed and theoretical distribution patterns.

The partition coefficient of a substance in a carefully defined two-phase system can also serve as an identifying physical constant, a fact which has been successfully exploited by UDENFRIEND and his collaborators in establishing the specificity of analytical procedures for several indole compounds.

BOGDANSKI et al. (1956) identified 5-HT in brain of rat and rabbit by means of countercurrent distribution, which was carried out in glass-stoppered centrifuge tubes using the solvents n-butanol and 0.55 M pyrophosphate buffer pH 9.4; each solvent was previously saturated with respect to the other. After distribution, the partition coefficient of 5-HT in each tube was determined by measurement of its concentration in each phase. Analysis of the butanol phase was made after extracting the 5-HT into 0.1 N HCl, following the addition of two volumes of heptane (see p. 99). The 5-HT in the buffer phase and in the 0.1 N HCl was determined from its fluorescence at 550 mμ, after the solution had been made 3 N with respect to HCl. Countercurrent distribution curves obtained for the apparent 5-HT extracted from rabbit brain are shown in Fig. 7. The location of the peaks and the shape of the curves were found to be almost identical; both curves were almost the same as that calculated from the measured partition

coefficient of authentic 5-HT. It was also apparent that no bufotenine occurs in the brain. To obtain additional evidence that the 450 mμ fluorescence represented 5-HT, the distribution of the apparent 5-HT between the two phases was measured in each tube. Except in a few of the last tubes the ratio of the amount of material in the butanol phase to the total amount in both phases was constant, and almost identical with that found for authentic 5-HT measured at the same time. A small amount of non-5-HT material was found in a few of the last tubes. The theoretical amount of 5-HT that should be present in these tubes was calculated from the measured partition coefficient of 5-HT by application of the binomial expansion in the manner described by WILLIAMSON and CRAIG (1947) and was found to be about 9 per cent. However, the same tubes had been found by analysis to contain a total of about 16 per cent of the total apparent 5-HT. Therefore, non-5-HT material represented about 7 per cent of the material measured in brain as 5-HT.

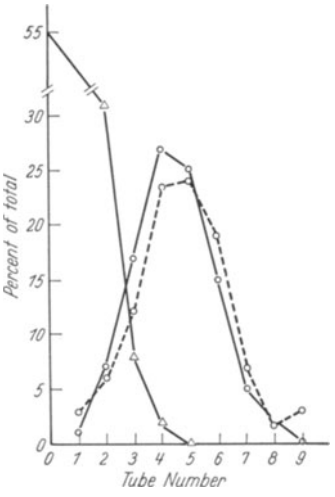


Fig. 7. Countercurrent distribution curves of authentic and apparent 5-HT extracted from brain of rabbit. ●——● represents authentic 5-HT; ○——○ represents apparent 5-HT in brain; ▲——▲ represents authentic bufotenine. From BOGDANSKI et al. (1956).

The above countercurrent distribution technique was also used by SJOERDSMA et al. (1959) to identify T isolated from human urine (see p. 94). Countercurrent distribution was carried out with benzene and 0.25 M borate buffer, pH 9.4 as solvents. After distribution, the partition coefficient of T was determined by measurement of its concentration in each phase. The organic phas was assayed after extraction of the T into 0.1 N HCl. The T from both phases was determined by spectrophotofluorometry after readjusting to pH 9—10. From the countercurrent distribution curves obtained and corresponding theoretical calculations, it was found that only 5 per cent of the material measured as T consisted of non-T substances. Further, N-methyl analogues of T, such as N-methyl-5-HT and bufotenine, could be excluded as urinary metabolites, since their countercurrent distribution curves were clearly different from that of T.

V. Paper chromatography

Although a wide variety of analytical methods are presently available for indole analysis, paper chromatography still retains a unique position in this field. Historically, it has been the most widely used method for separation and identification of indoles. Practically, it requires minute amounts of starting material, and is simple to perform without elaborate equipment.

Paper chromatography of indole compounds has been discussed by several workers, e.g. ERSPAMER (1954), DALGLIESH (1955, 1956), PROCHÁZKA (1958), ARMSTRONG et al. (1958), JEPSON (1960) and SANDLER (1963).

For the principles and practice of paper chromatography the reader is referred to the many excellent monographs available, e.g. HAIS and MACEK (1958), BLOCK et al. (1958), LINSKENS (1959), SMITH (1960), MACEK (1961) and CONSDEN (1961).

1. Chromatography paper

A large variety of chromatography papers has been used in indole analysis. However, the literature indicates that Whatman No. 1 and No. 4 have been used

more than any other kind for qualitative work. The former is a "medium rate" paper; the latter is "fast", about two times that of No. 1. In recent years Whatman No. 1 has in some countries been replaced by Schleicher & Schüll 2043b, which is a standard filter paper which is usable in practically all types of chromatographic separations. Whatman No. 5 and No. 42 are "slow" papers. For isolation purposes a thick paper may be required, e.g. Whatman No. 3 or 3 MM (see p.71).

2. Solvent systems

A large number of solvent systems are used, far too many to list in this discussion. Here, only some of the most widely used systems will be given:

a) Butanol-acetic acid-water (4:1:5)

This is a good general solvent for indole compounds and has been used by many workers (e.g. ERSPAMER 1955, DALGLIESH 1956, ARMSTRONG et al. 1958, MCISAAC and PAGE 1959, ERSPAMER and NOBILI 1962a, b). In this solvent chromatograms are usually run overnight.

b) Isopropanol-ammonia-water (8:1:1)

This is an excellent solvent for indoles by virtue of its high resolution power. It is remarkably little affected by the presence of inorganic ions, and solutions so chromatographed need no prior desalting.

The R_f values obtained with this solvent are rather dependent on the ammonia concentration and this must be carefully controoled.

This solvent system has been used by ARMSTRONG et al. (1958), MCISAAC and PAGE (1959), ERSPAMER and NOBILI (1962a, b) and others. Chromatograms are usually run overnight.

c) Potassium chloride

Potassium chloride, 20 per cent water solution.

This solvent, which was applied to indole analysis by DALGLIESH (1956), allows rather rapid separation of many indole compounds; a run usually requires only 3—4 hours. Compact spots are usually obtained, and the R_f values are remarkably constant.

This solvent has ben used by several other workers, e.g. ARMSTRONG et al. (1958) and ERSPAMER and NOBILI (1962a, b).

d) Benzene-propionic acid-water (100:70:5)

This solvent, which was introduced by ARMSTRONG et al. (1958), is particularly useful for the separation of indole acids.

The four above systems have been chosen for mention here because they give the best chromatograms in general indole investigations and screening operations. Furthermore, a larger number of indole compounds have been run with these solvents than with any other systems. A large number of other solvent systems have been described for smaller groups of indole compounds; some of these are given in Table 4.

For two-dimensional chromatography of indole compounds several combinations of solvent systems have been recommended. Generally, a basic solvent and a neutral or acidic solvent will give the best separations. JEPSON (1955) recommends isopropanol-ammonia-water (20:1:2) in the first dimension, followed by butanol-acetic acid-water (12:3:5) in the second dimension. This combination

Table 4a. *R_f values of indolealkylamines and related compounds*

Compound	Solvent system								
	a	b	c	d	e	f	g	h	i
Indolealkylamines:									
Tryptamine	0.70	0.72	0.48		0.94	0.76	0.90	0.44	
1-Methyltryptamine	0.70	0.84				0.75			
2-Methyltryptamine	0.75	0.87				0.80			
α-Methyltryptamine	0.78	0.88				0.82			
N-Methyltryptamine	0.73	0.88				0.76		0.48	
N-Ethyltryptamine	0.83	0.93	0.60			0.79			
α-Ethyltryptamine	0.84	0.92	0.52			0.86			
N,N-Dimethyltryptamine	0.74	0.92	0.60			0.82		0.48	
α,α-Dimethyltryptamine	0.82	0.90	0.60			0.87			
β,β-Dimethyltryptamine	0.81	0.92	0.58			0.79			
N,N-Diethyltryptamine	0.85	0.98	0.62			0.83			
4-Hydroxytryptamine	0.60	0.66	0.36			0.75			
4-Hydroxytryptamine glucuronide	0.18	0.05	0.65						
N,N-Dimethyl-4-hydroxytryptamine (Psilocin)	0.74								
5-Hydroxytryptamine	0.48	0.69	0.37		0.86	0.70	0.62	0.20	
5-Hydroxytryptamine glucuronide	0.14	0.09	0.66						
5-Methoxytryptamine	0.64	0.76	0.30			0.72	0.80	0.34	
N-Methyl-5-hydroxytryptamine	0.49	0.73			0.83	0.72			
Bufotenine	0.62	0.92			0.91	0.79			
Bufotenidine	0.59	0.20			0.15				
Bufothionine	0.51	0.24	0.43		0.34	0.72			
Dehydrobufotenine	0.76	0.46			0.09				
N-Acetyl-5-hydroxytryptamine	0.81	0.75			0.86				
Melatonin	0.90	0.83							
N-Acetyl-5-methoxy-6-hydroxy-tryptamine	0.67								
N-Acetyl-5-methoxy-6-hydroxy-tryptamine glucuronide		0.22							
N-Acetyl-5-methoxy-6-hydroxy-tryptamine sulphate		0.55							
6-Hydroxytryptamine	0.44	0.53	0.33			0.72			
6-Methoxytryptamine	0.65	0.75	0.30			0.72			
7-Hydroxytryptamine	0.53	0.46				0.76			
5,6-Dimethoxytryptamine	0.50	0.65	0.24			0.62			
Indole amino acids:									
Tryptophan	0.37	0.34	0.54	0.07	0.08	0.56			0.77
N-Acetyltryptophan	0.83	0.53	0.74	0.42		0.70			0.80
N-Methyltryptophan	0.60	0.37				0.65			0.95
4-Hydroxytryptophan	0.29	0.30	0.67						
4-Hydroxytryptophan glucuronide	0.08								
5-Hydroxytryptophan	0.22	0.27	0.43		0.03	0.40			0.46
5-Hydroxytryptophan glucuronide	0.08		0.70						
N-Acetyl-5-hydroxytryptophan	0.75		0.66				0.23		
N-Acetyl-5-hydroxytryptophan glucuronide	0.23		0.70						
N-Acetyl-5-acetoxytryptophan	0.81		0.75				0.21		
5-Methoxytryptophan	0.27	0.32	0.36	0.06		0.52			0.76
Indole carboxylic acids:									
Indole-3-carboxylic acid	0.83	0.35	0.36	0.71		0.93			0.60
glyoxylic acid	0.67	0.48	0.42	0.11		0.72			0.60
glyoxylamide	0.76	0.85	0.11	0.49					
glycolic acid	0.82	0.39	0.74	0.18		0.56			0.50
acetic acid	0.85	0.47	0.63	0.72	0.12	0.84	0.52	0.84	0.75
acetamide	0.77	0.85	0.51	0.67		0.90			0.95

Table 4a (continued)

Compound	Solvent system								
	a	b	c	d	e	f	g	h	i
Indoleaceturic acid	0.76	0.48	0.65	0.29		0.65	0.46	0.58	0.82
N-(Indole-3-acetyl)-									
α-alanine	0.84	0.60	0.71	0.47					
β-alanine	0.82	0.51	0.70	0.46					
β-aminoisobutyric acid	0.86	0.60	0.71	0.66					
γ-aminobutyric acid	0.84	0.52	0.67	0.56					
aspartic acid	0.72	0.11	0.78	0.14		0.55			0.48
asparagine	0.59	0.33	0.72	0.17		0.60			0.78
glutamic acid	0.78	0.12	0.78	0.19		0.43			0.51
glutamine	0.65	0.37	0.70	0.19		0.58			0.82
β-(Indole-3)-									
pyruvic acid	0.80			0.41					
lactic acid	0.78	0.48	0.61	0.29		0.71			0.63
propionic acid	0.86	0.53	0.44	0.80	0.19	0.89			0.78
acrylic acid	0.83	0.45	0.15	0.73		0.94			0.70
4-Hydroxyindoleacetic acid	0.87	0.55	0.42						
4-Hydroxyindoleacetic acid glucuronide	0.24								
5-Hydroxyindole-									
2-carboxylic acid	0.72	0.29	0.24	0.17					
3-carboxylic acid	0.70	0.19	0.30	0.13					
3-acetic acid	0.72	0.27	0.51	0.14	0.03	0.61	0.16	0.60	0.46
3-acetic acid-5-sulphate ester	0.28	0.05	0.70						
3-acetic acid glucuronide	0.24								
5-Hydroxyindoleaceturic acid	0.84	0.23							
β-(5-Hydroxyindole-3)-lactic acid	0.53	0.29	0.50	0.03					
5-Methoxyindole-									
2-carboxylic acid	0.82	0.45	0.20	0.76					
3-carboxylic acid	0.82	0.32	0.29	0.71					
3-acetic acid	0.83	0.39	0.49	0.66		0.82	0.44	0.78	0.78
β-(5-Methoxyindole-3)-lactic acid	0.70	0.44	0.50	0.27					
6-Hydroxyindoleacetic acid	0.76	0.14				0.58			
7-Hydroxyindoleacetic acid	0.84	0.13				0.68			

Table 4b. Solvent Systems

System	Composition	R_f values given by:
a	Butanol-acetic acid-water (4:1:5), see p. 83	ARMSTRONG et al. (1958), JEPSON (1960), MCISAAC and PAGE (1959), JEPSON et al. (1962), ERSPAMER and BERTACCINI (1962), ERSPAMER and NOBILI (1962).
b	Isopropanol-ammonia-water (8:1:1), see p. 83	
c	Potassium chloride, 20% water sol., see p. 83	ARMSTRONG et al. (1958), JEPSON (1960), ERSPAMER and BERTACCINI (1962), ERSPAMER and NOBILI (1962).
d	Benzene-propionic acid-water (100:70:5), see p. 83	ARMSTRONG et al. (1958).
e	Methyl ethyl ketone-2 N NH_3 (2:1)	MCISAAC and PAGE (1959).
f	Butanol-pyridine-water (1:1:1)	JEPSON (1960).
g	Butanol-methylamin 25% (8:3)	ERSPAMER (1955), ERSPAMER and NOBILI (1962b)
h	Butanol, saturated with 1 N HCl	ERSPAMER (1955).
i	Phenol-ammonia	JEPSON (1960).

gives excellent separation of most indoles and is sepecially useful in the analyses of urine or tissue extracts. For a detailed description of this system, the reader is referred to JEPSON (1960).

DALGLIESH (1956) recommends the organic layer of butanol-acetic acid-water (4:1:5) for the first dimension and 20 per cent KCl for the second dimension.

3. Location of spots

a) Colour reactions

All the colour reactions mentioned above (p. 77) are useful in paper chromatography.

α) Aldehyde reagents (see. p. 77). p-Dimethylaminobenzaldehyde in dilute HCl (EHRLICH's reagent) is by far the most important of these reagents. As a spray reagent it is usually employed in an acid alcohol solution (2 g of p-dimethylaminobenzaldehyde dissolved in a mixture of 80 ml of 95 per cent ethanol and 20 ml of 6 N HCl). It can also be made up with acetone and used as a dip reagent. JEPSON (1960) prefers the latter and uses a mixture of 1 volume of 10 per cent p-dimethylaminobenzaldehyde in conc. HCl and 4 volumes of acetone. This mixture is prepared just before use.

Spots appearing with p-dimethylaminobenzaldehyde range in colour from blue-green through blue to bright pink. The rate of development of these colours varies considerably with different indole compounds; some compounds may develop maximal colour within 5 minutes while others may require more than 24 hours (see ARMSTRONG et al. 1958). When working with this reagent it is very important to note the *initial* colours, *changes* in colour, and the *permanance* of the colours. The sequence of colours in the development of the spots may be quite characteristic, and is therefore useful in the identification of a given indole compound. Sometimes a spot may appear strongly, fade almost to disappearance and reappear later with an enhanced or different colour. 5-HIAA may be taken as an example: its colour sequence is a rapid blue spot, changing to faint grey, and later to stable blue over a 30 minute period. Hence, as pointed out it is advisable to inspect the chromatograms at intervals over the first hour and again the next day.

p-Dimethylaminocinnamaldehyde, which was used by HARLEY-MASON and ARCHER (1958), is prepared for spraying by dissolving 2 g of the aldehyde in a mixture of 100 ml of 6 N HCl and 100 ml of 95 per cent ethanol. For dipping, a 1 per cent solution of the aldehyde in conc. HCl is diluted with 4 volumes of acetone just before use. Most 5-hydroxyindoles react with this reagent to give blue colours. 4-Hydroxyindoles only give grey, 6-hydroxyindoles a grey-blue and 7-hydroxyindoles a green colour.

Cinnamaldehyde is usually sprayed as a 1 per cent solution in methanol. The chromatograms are then exposed to HCl vapours in a glass container until the spots are clearly developed (MÜLLER 1953).

β) Xanthydrol reagent (see. p. 78). A 0.1 per cent solution of xanthydrol in 95 per cent ethanol containing 5 per cent conc. HCl is used as a spray reagent. The solution is prepared fresh on the day of use. Colour appears on drying the paper at room temperature. The paper may also be sprayed with an alcoholic xanthydrol solution, and after drying placed over conc. HCl in a sealed container.

For quantitative work, the coloured material can be eluted from the paper with methanol, the latter evaporated, and the residue taken up in 6 N HCl and the absorbancy determined (DICKMAN and CROCKETT 1956).

γ) 1-Nitroso-2-naphtol reagent (see p. 79). The paper is first sprayed with a 0.1 per cent 1-nitroso-2-naphtol solution in 95 per cent ethanol. After drying the paper is sprayed with a freshly prepared mixture of 25 ml of 2 N HCl and 1 ml of 2.5 per cent $NaNO_2$. The 5-hydroxyindoles appear as violet spots on a

faint yellow background. At least 5 to 10 μg of compound are needed with this reagent (UDENFRIEND and WEISSBACH 1963).

4-Hydroxyindoles give a weak yellow and 6- and 7-hydroxyindoles a weak brown colour with this reagent (JEPSON et al. 1962).

δ) Diazo reagents (see p. 79). Diazotized sulphanilic acid is used in both acid (van den Bergh reaction) and in sodium carbonate (PAULY's reaction). The *acid* reagent is made up as follows:

a) Sulphanilic acid, 9 g, conc. HCl 90 ml, water 900 ml . . . . 10 vol.
b) Sodium nitrite, 5 per cent in water . . . . . 1 vol.

The two solutions are mixed and immediately placed in an ice-bath for 5 minutes. Then the remaining nitrous acid is destroyed with solid ammonium sulphamate. When sprayed with this reagent 6-hydroxy- and 6-methoxyindoles react *immediately* and specifically to give brilliant red colours. 4-, 5- and 7-Hydroxyindoles yield yellow-brown to purple colours which develop rather slowly.

Diazotized sulphanilic acid in *alkaline* solution is prepared as follows:

a) Sulphanilic acid, see above . . . . . 1 vol.
b) Sodium nitrite, see above . . . . . 1 vol.
c) Sodium carbonate, 10 per cent in water . . . . . 2 vol.

When required the stock solutions of a and b are mixed and allowed to stand for 5 minutes at room temperature. Solution c is then added carefully as the mixture vigorously effervesces. The reagent is used immediately and is usually sprayed on the chromatograms. 4-Hydroxyindoles are coloured brown-violet, 5-hydroxyindoles red-brown, 6-hydroxyindoles purple, and 7-hydroxyindoles grey-brown (JEPSON et al. 1962).

Diazotized p-nitroaniline may be used in the same way as diazotized sulphanilic acid. A suitable reagent is made up as follows:

a) p-Nitroaniline, 0.1 per cent in 1 N HCl . . . . . 1 vol.
b) Sodium nitrite, 0.2 per cent in water . . . . . 1 vol.
c) Potassium carbonate, 10 per cent in water . . . . . 2.5 vol.

The separate reagents are kept in stock solutions at 5°. For the *acid* reagent a and b are mixed and kept cool for a further 5 minutes and then sprayed on the paper. When the *alkaline* reagent is required, an additional spray is made with solution c. If an alkaline reagent is to be used directly, c is added to the cooled mixture of a and b and the paper sprayed at once. This spray reagent must be prepared fresh and used immediately as it deteriorates rapidly. A volume of 10 ml is sufficient for one 25×25 cm chromatogram.

Diazotized p-nitroaniline in *acid* also gives an immediate intense red colour with 6-hydroxyindoles. 4-, 5- and 7-Hydroxyindoles react more slowly yielding colours which vary from yellow to red-brown. In the *alkaline* version 6-hydroxyindoles give purple colours, 4-hydroxyindoles grey-blue, 5-hydroxyindoles pink, and 7-hydroxyindoles green-blue colours.

p-Nitrobenzenediazoniumtetrafluoroborate is used as a 0.5 per cent aqueous solution, which is stable at room temperature. 5-Hydroxyindoles give red to brown-red colours. Simple indoles, such as TP, T and IAA, show yellow to orange colours. A further differentiation may be obtained by subsequently spraying with a 10 per cent sodium carbonate solution.

2-Chloro-4-nitrobenzenediazonium naphtalene-2-sulphonate (NNCD reagent) is usually employed as a freshly prepared 0.1—0.3% solution in 0.1 N HCl. After spraying, the chromatograms are allowed to dry in air. Simple indoles, e.g. T, IAA, N-methyl-T and N,N-dimethyl-T react to give orange colours. 4- and 5-Hydroxyindoles result in red colours (ERSPAMER 1955, ERSPAMER and NOBILI 1962a).

ε) Other colour reactions. Several other colour reactions have been used for the location of indole compounds on chromatograms: descriptions of these are to be found in the monographs by JEPSON (1960) and CLOTTEN and CLOTTEN (1962) and in the publications of DALGLIESH (1955, 1956).

b) Fluorescence reactions

Although most indoles *adsorbed on paper* do not fluoresce, it is always advisable to examine the untreated chromatogram in ultraviolet light, and again after each colour reagent has been applied. After reacting with a reagent, ultraviolet light can sometimes provide a more sensitive means for examining weak spots on chromatograms than visible light.

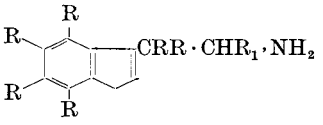
However, when sprayed with a number of reagents indole compounds show fluorescence (see p. 74) and may thus be detected on developed chromatograms.

α) HCl-fluorescence. When sprayed with 0.1 N HCl and examined under an ultraviolet lamp 5-HT and other 5-hydroxyindoles emit a pink fluorescence. This fluorescence is fairly specific, but not very sensitive (5—10 μg required per spot). However, it may be useful as a location method when chromatography or electrophoresis on paper are used as isolation procedures (see p. 71).

β) Ninhydrin-acetic acid fluorescence (see. p. 74). The chromatograms are dipped in or sprayed with a freshly prepared reagent of the following composition:

Ninhydrin, 0.1 per cent in acetone	9 vol.
Acetic acid, conc.	1 vol.

After drying the paper, it is heated at 110° for 2 minutes. Susceptible tryptamines show an intense greenish-blue fluorescence in ultraviolet light which is detectable at concentrations as low as 0.01 μg per cm^2 paper. This test, which is specific for certain tryptamines, depends upon the formation of 3,4-dihydro-3-carboline (JEPSON 1960). A strongly positive reaction specifically indicates a compound with a tryptamine structure not more substituted than the following:



Alkyl groups can be introduced at any or all of the positions shown in the above structure, without any diminution in fluorescence. Substitution of any of the hydrogen atoms shown in the structure abolishes fluorescence. R_1 cannot be —COOH (tryptophan), but may well be —CH_2OH (tryptophanol). With tryptamines bearing a substitutent at position 6 the fluorescence colour is yellowish.

Tryptamines, N-alkyltryptamines and N,N-dialkyltryptamines, which react similarly with p-dimethylaminobenzaldehyde reagent, can be distinguished by their response to the ninhydrin-acetic acid reagent. Tryptamines-(NH_2) give strong visible colour and strong fluorescence, N-alkyltryptamines-(NHR) strong visible colour but little fluorescence, and N,N-dialkyltryptamines-(NRR) no visible colour and no fluorescence.

γ) Formaldehyde-HCl-fluorescence (see p. 74). The chromatograms are sprayed with the following mixture:

Formaldehyde (35—40 per cent)	1 vol.
HCl, conc.	1 vol.
Water	2 vol.

and then dried at 90°. Most indoles substituted at the 3-position show orange to brown visible colours with characteristic yellow or orange fluorescence (PRO-

CHAZKA 1953). For structural requirements of the molecules for this reaction, see p. 75.

δ) Formaldehyde-K_2CrO_7-fluorescence (see. p. 75). SHEPHERD et al. (1953) have described a similar fluorescence reaction but use potassium dichromate instead of hydrochloric acid. Their reagent is the following:

Potassium dichromate, 0.1 per cent in water . . . . . 9 vol.
Formaldehyde (35—40 per cent) . . . . . 1 vol.

After spraying, the chromatograms are heated at 100—110° for 5 minutes. When examined in ultraviolet light 5-HT shows a golden-yellow fluorescence immediately after heating, and this persists for days; amounts as small as 0.2 μg of 5-HT can be detected. The behaviour of bufotenine and bufotenidine is quite similar to that of 5-HT. T gives a bright yellow fluorescence if more than 2.5 μg are present, but this develops slowly. TP likewise gives a yellowish fluorescence if 2.5 μg are present, while 5-methoxy-T gives yellow-orange fluorescence if similar amounts are present.

ε) Iodate-acetic acid-fluorescence (see p. 75). When chromatograms are sprayed with a saturated solution of potassium iodate in 50 per acetic acid (about 2.5 per 100 ml) and then heated at 60° for 5 minutes, TP and T form tryptochrome which shows a brown-red visible colour with intense orange fluorescence (EGELHAAF 1957). 0.1 μg of TP may be detected.

4. R_f values of indolealkylamines and related compounds

In Table 4 R_f values are given for a number of biologically important indolealkylamines and related compounds in different solvents.

5. Quantitative paper chromatography

Although paper chromatography cannot, by its very nature, be more than semi-quantitative, it may be necessary to use it when no other quantitative procedure exists.

GOBLE et al. (1955) used the fluorescence reaction of JEPSON and STEVENS (1953) for 5-HT measurements, comparing the unknown spots with standards. RODNIGHT (1956) used a similar procedure with p-dimethylaminobenzaldehyde spots for the determination of urinary 5-HT and T. However, these spots are not ideal for this purpose, as colours may change and the speed at which they appear may vary depending on whether the indoles are present together with urine or in pure solution (see p. 86).

SCHREIER and GAEDTKE (1956) measured 5-HT and IAA in pure solutions by spraying the chromatograms with a mixture of equal parts of 10 per cent xanthydrol in methanol and acetic acid. The spots were then cut out, eluted with a mixture of acetic acid and methanol (2:1) and the colour read at 580 mμ. GAEDTKE and SCHREIER (1956) used the same technique for determination of 5-HT in urine, but acetylated the 5-HT before chromatography.

CORREALE (1955) used paper chromatography for the determination of 5-HIAA. The spots were eluted with water and the 5-HIAA determined in the eluates by using the p-dimethylaminobenzaldehyde reaction.

CARCASONA et al. (1959b) separated 5-HTP, 5-HT and 5-HIAA by paper chromatography, sprayed the papers with p-dimethylaminobenzaldehyde, 1-nitroso-2-naphtol or diazotized sulphanilic acid. The papers were then made transparent with a mixture of α-bromonaphtalene and paraffin oil and the spots scanned densitometrically.

VI. Thin layer chromatography

Whereas paper chromatography has proven an effective and useful tool for separation of indole compounds, an alternative method — thin layer chromatography — providing equivalent resolution has been recently developed.

The principles and techniques of thin layer chromatography will not be treated here. Excellent reviews have been published by WOLLISH et al. (1961) and DEMOLE (1959), and the various techniques are comprehensively reviewed in the books by STAHL (1962), RANDERATH (1962), TRUTER (1963) and BOBBITT (1963).

Table 5. *R_f values of indole compounds on silica gel G layers*

Compound	R_f values in Solvent 1	R_f values in Solvent 2	References
Indole	0.84	0.73	1
3-Hydroxymethylindole	0.84	0.45	1
Indoleacetic acid	0.31	0.28	1
Indolepropionic acid	0.38	0.34	1
Indolebutyric acid	0.40	0.38	1
Indoleacrylic acid	0.33	0.29	1
5-Hydroxyindoleacetic acid	0.19	0.04	1
Tryptamine	0.77	0	1
5-Methoxytryptamine	0.62	0	2
5-Hydroxytryptamine	0.65	0	1, 2
Melatonin	0.89	0.26	2
N-Acetyl-5-hydroxytryptamine	0.90	0.08	2
Tryptophan	0.23	0	1
5-Methoxytryptophan	0.28	0	1
5-Hydroxytryptophan	0.14	0	1
Bufotenine	0.90	0	2
N-Acetyltryptophan	0.56	0.07	2

Solvent 1: Methyl acetate-isopropanol-25% ammonia (9:7:4). Solvent 2: Chloroform-96% acetic acid (19:1).

References to Table 5. [1] STAHL and KALDEWEY (1961). [2] HANSON (1964).

Thin layer chromatography, a technique essentially complementary to paper chromatography, may offer such advantages as:

1. Small, sharply defined spots, frequently better than those which can be obtained with the same compounds by paper chromatography.
2. Extraordinary rapid development.
3. The results can be readily applied to large-scale preparative separations on columns.
4. There are no limitations on the reagents which can be used for localization of the spots.

The method can frequently be used with amounts ten to hundred times less than those needed for paper chromatography.

These advantages are all well illustrated by the results obtained with indole compounds. Thin layer chromatography here is about one hundred times as sensitive as paper chromatography, a feature which is very important in studies of biological materials.

STAHL and KALDEWEY (1961) and HANSON (1964) have used neutral, unbuffered silica gel *G* (Merck) as an adsorbent, and this material gives a good separation with many indole compounds. The chromatoplates are of the size 20×20 cm and each development is carried to a distance of about 10 cm. Methyl acetate-isopropanol-25% ammonia (9:7:4) and chloroform-96% acetic acid (19:1) were used as solvent systems, either singly, or combined for two-dimensional chromatography. In these systems the development usually takes about 30 minutes. The indoles are located with either of the following reactions (STAHL and KALDEWEY 1961):

Van Urk reaction. Reagent: 1 g of p-dimethylaminobenzaldehyde is dissolved in a mixture of 50 ml of 25% HCl and 50 ml of 96% ethanol. Each 20×20 cm chromatoplate is sprayed with about 10 ml of the reagent. Ammonia from the developing solvent should be removed by first warming the chromatoplate to 50° for 5 minutes. After the sprayed chromatoplate has been warmed to 50° for 20 minutes it is exposed to the vapours from aqua regia. Indole compounds then appear as blue, violet or red spots. This reaction, which was found to be the most specific, allows a detection of 0.05 to 0.1 μg of most indole components.

Prochazka fluorescence reaction. Reagent: 10 ml of 35% formaldehyde is mixed with 10 ml of 25% HCl and 20 ml of 96% ethanol. Again the chromatoplate is sprayed with 10 ml of the reagent and after about 5 minutes heated to 100°. Indole compounds form yellowish brown visible spots which have an intense orange fluorescence in ultraviolet light (see p. 74). Exposure of the sprayed plate to the vapours of aqua regia, or simple storage in air for 24 hours results in an intensification of the fluorescence. This reaction, which was found by STAHL and KALDEWEY (1961) to be the most sensitive, allows a detection of 0.005 to 0.01 μg of most indole compounds.

Instead of the above van Urk reagent, the p-dimethylaminobenzaldehyde reagent with acetone (described in the above section on paper chromatography, p. 86) is equally satisfactory for thin layer chromatography.

HANSON (1964) has used thin layer chromatography for the rapid analysis of carcinoid urines. A small volume (2—3 ml) of acidified urine is extracted with ether or ethyl acetate. The extract is dried with anhydrous sodium sulphate and evaporated. The residue is taken up in 0.1—0.2 ml of ethanol and 5 μl of the extract is spotted on a chromatoplate. After development in the above described methyl acetate-isopropanol-ammonia system, the plate is sprayed with p-dimethylaminobenzaldehyde-acetone reagent and dried. It is then inspected for the presence of 5-HIAA and other indole compounds. In this way the chromatographic analysis of a urine sample can be completed in about 1 hour.

Table 5 summarizes R_f values for some biologically important indole compounds, as resolved on neutral silica gel.

VII. Gas chromatography

The failure to apply gas-liquid chromatography to indole analysis apears to be based on technical features presently inherent in the instrumentation involved. For current gas chromatography columns, only a very small volume may be applied (1—10 μl); hence, very high indole concentrations must be achieved

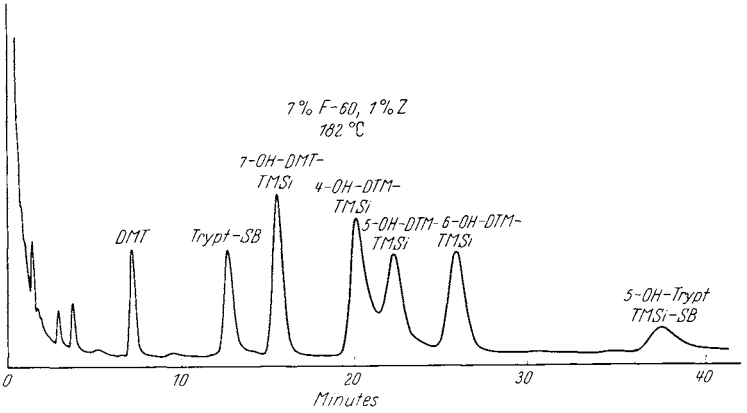


Fig. 8. Gas chromatographic separation of a mixture of closely related T derivatives. The compounds are N,N-dimethyl-T (DMT), The acetone condensation product (Schiff base) of T (TRYPT-SB); 7-trimethylsilyloxy-N,N-dimethyl-T (7-OH-DMT-TMSi); 4-trimethyl-silyloxy-N,N-dimethyl-T (4-OH-DMT-TMSi); 5-trimethyl-silyloxy-N,N-dimethyl-T (5-OH-DMT-TMSi); 6-trimethylsilyloxy-N,N-dimethyl-T (6-OH-DMT-TMSi); acetone condensation product of 5-trimethylsilyloxy-T (5-OH-TRYPT-TMSi-SB). From HOLMSTEDT et al. (1964)

for suitable detection. This is in contrast to spectrophotofluorometrical techniques whereby indoles may be detected at the 0.1 μg level in 1 ml of fluid. However, in situations where high concentrations can be obtained, gas-liquid chromatography permits not only qualitative resolution, but quantitative estimation as well. A final outstanding feature well documented for gas-liquid chromatography is the ability to resolve closely related indoles.

The principles and practice of gas-liquid chromatography will not be discussed here; for this the reader is referred to the many manuals available (e.g. KEULEMANS 1959, BURCHFIELD and STORRS 1962).

SWEELEY and WILLIAMS (1961) have used gas-liquid chromatography to separate and quantitatively estimate aromatic acids-including IAA and 5-HIAA — from human urine after conversion of the acids to their methyl esters.

FALES and PISANO (1962) were able to separate microgram quantities of a number of biogenic amines including T, dimethyl-T, 5-methoxy-T, 5-HT, N-acetyl-T and melatonin.

HOLMSTEDT et al. (1964) have studied the separation and identification of a number of tryptamine related indole bases by gas-liquid chromatography. The indoles studied were N,N-dimethyl-T, N,N-diethyl-T, T, 5-methoxy-T, 5-methoxy-N,N-dimethyl-T, 5-HT, 4-hydroxy-N,N-dimethyl-T, 5-hydroxy-N,N-dimethyl-T, 6-hydroxy-N,N-dimethyl-T and 7-hydroxy-N,N-dimethyl-T. These compounds were chromatographed both directly and as the trimethylsilyl ethers. The latter derivatives provided a satisfactory separation of the positional isomers 4-, 5-, 6- and 7-hydroxy-N,N-dimethyl-T (see Fig. 8).

By means of their gas-liquid chromatographic technique the latter workers were able to identify 5-methoxy-N,N-dimethyl-T as the major indole base of *epená*, a South American snuff with reported hallucinogenic properties.

VIII. Paper electrophoresis

Electrophoresis on filter paper offers another method, complementary to paper chromatography, for the separation and identification of minute quantities of closely related indole compounds.

According to the potential gradient used distinction is made between low and high voltage electrophoresis. *Low voltage electrophoresis* is carried out at voltages not exceeding 300—400 volts or a potantial gradient of not more than 10 volts per cm in the direction of the migration. In *high voltage electrophoresis*, which is the most important recent development in zonal electrophoresis, much higher voltages (1000 to 10,000 volts) and potantial gradients (20 to 200 volts per cm) are employed.

The theory and practice of paper electrophoresis will not be treated here; for this the reader is referred to the books by LEDERER (1955), BLOCK et al. (1958), RIBEIRO et al. (1961) and MORRIS and MORRIS (1963) for techniques of low voltage electrophoresis, and to EFRON (1960) and CLOTTEN and CLOTTEN (1962) for comprehensive reviews of high voltage electrophoresis.

A number of low voltage electrophoretic systems have been described for the analysis of indole compounds. Plant extracts were analyzed for indoles by DENFFER et al. (1952), who used a phosphate buffer of pH 7 and a potential of 110 volts for 8 hours. TP and IAA separated without difficulty in this system. MÜLLER (1953) also studied these compounds by paper electrophoresis.

LANGEMANN and KÄGI (1956) used low voltage electrophoresis for the separation of 5-HT and 5-HIAA in a case of carcinoid. The electrophoresis was run for 6 to 10 hours in 0.2 M acetate buffer pH 4.8 or 0.067 M barbital buffer pH 8.6 at a voltage of 220 volts.

ROBERTSON and ANDREWS (1961) used low electrophoresis in the detection and estimation of 5-HT in human plasma. A 0.1 ml plasma sample was applied to the paper strip which was run in a citrate buffer pH 3.8 at a potential gradient of 10 volts per cm for 6 hours. After elution of the appropriate area with Tyrode solution 5-HT was determined by biological assay.

CARCASONA et al. (1960) separated 5-HTP, 5-HT and 5-HIAA by using barbital buffer pH 8.6, phosphate buffer pH 7.0 or acetate buffer pH 5.05 and a potential gradient of 3.5 volts per cm. In these systems 5-HT migrates towards the cathode and 5-HIAA towards the anode, while 5-HTP migrates very little. The time necessary for optimal separation was 8 to 12 hours. The paper strips were sprayed with p-dimethylaminobenzaldehyde reagent and, after drying, made transparent with a mixture of α-bromonaphtalene and paraffin oil. The colour intensity of the spots was then determined by densitometry.

The time necessary for separation of small molecules by low voltage electrophoresis is usually long and during this time substances not only migrate according to their electrical charge but also diffuse into the surrounding media. This often results in large, faint, overlapping spots and consequently poor resolution. For this reason is often proves advantageous to use a high voltage gradient to complete the process of migration rapidly. The resulting heat which is generated requires special arrangements for cooling, which somewhat complicates the equipment, but the definition of the spots and the degree of resolution is unequalled by any alternative methods.

It is thus apparent that there are two major advantages of high voltage electrophoresis for the separation of small molecules: The separation is rapid, and diffusion is minimized. To these can further be added that specimens usually need not be desalted. Urine and plasma samples can be applied directly to the paper strips without any prior preparation.

A large number of high voltage electrophoretic systems have been described for the analysis of indole compounds (see CLOTTEN and CLOTTEN 1962).

Some of the most useful buffers for the separation of indole compounds commonly encountered in biological specimens are the following:

1. Borate buffer pH 10 (EFRON 1960).
2. Pyridine-acetate buffer pH 6.1 (EFRON 1960).
3. Citrate buffer pH 3.8 (ROBERTSON and ANDREWS 1961).
4. Pyridine-acetate buffer pH 3.6 (ZYLKA and KÖHLER 1957, STURM 1961).
5. Pyridine-acetate buffer pH 2.3 (GAYER 1956).
6. Formate-acetate buffer pH 1.9 (NÖLLER and STELGENS 1957).

The two first are the most effective for the separation of indolic acids from other indole compounds. The migration of most compounds is towards the anode in these systems. However, exceptions are 5-HT and other tryptamines which migrate towards the cathode. For separation of the amines the more acid buffers usually give better results.

With a high voltage applied, a very rapid separation of the compounds can be effected; e.g. a potential of 6000 volts often gives adequate separation in 30 minutes.

Following the electrophoretic separation the indole compounds may be located by any of the reagents described in the section on paper chromatography (p. 86).

After location of the individual indole compounds, their determination can be made either directly by densitometry or, after elution, by colorimetry, ultraviolet absorbancy, spectrophotofluorometry or biological assay.

If there is a large number of similarly charged compounds present in a specimen, overlapping of the spots often occurs. In such cases a separation in a second dimension may be very effective. This can be done by high voltage electrophoresis in one direction followed by chromatography performed in a perpendicular direction on the same paper. When such a two-way separation is made, electrophoresis should always be used for the first separation for three reasons: it automatically desalts the specimen, it effectively separate the compounds from

deproteinizing agents if such have been used, and it avoids leaving chromatographic solvents on the paper, as these may interfere with electrophoretic separation. For details on this combination of high voltage electrophoresis and chromatography, see EFRON (1960).

Specific high voltage electrophoretic methods for the determination of 5-HT in biological material have been described by NÖLLER and STELGENS (1957), ZYLKA and KÖHLER (1957) and STURM (1961) and for that of 5-HIAA by STURM (1962).

GAYER (1956) has been used a special technique for the detection of certain biogenic amines, including T and 5-HT. These amines are first converted to their tetraphenyl borates and are then separated by high voltage electrophoresis.

DEAVER et al. (1958) also applied a somewhat variant electrophoretic technique for the fractionation of mixtures containing indoles. Here use was made of the phenomenon of ionophoretic mobility equilibria as first described by DURRUM (see BLOCK et al. 1958).

C. Quantitative determination of specific compounds

In the following section a number of different methods for the quantitative determination of T and 5-HT, their precursors TP and 5-HTP and major metabolites IAA and 5-HIAA will be described.

I. Simple indoles

1. Tryptamine

T can be isolated from biological material by alkaline extraction into benzene, returned to an acid aqueous phase, and here determined either colorimetrically or fluorometrically.

a) Colorimetric determination

T forms a coloured product with xanthydrol which is very similar in optical characteristics to xanthydryltryptophan, i.e. showing an absorption maximum at 510 mμ (DICKMAN and WESCOTT 1954) and can thus be determined by the xanthydrol methods described for TP (p. 96) and IAA (p. 98).

b) Spectrophotofluorometric determination

Native fluorescence of T (SJOERDSMA et al. 1959). The following method is based on the fluorescence at 360 mμ shown by T when activated at 285 mμ and it is well suited for the determination of T in human urine:

A urine sample of 15 ml in a 50 ml glass-stoppered centrifuge tube is alkalinized to pH 11—14 by dropwise addition of 10 N NaOH. 30 ml of benzene are added, the tube is shaken for 20 minutes on a shaking machine and then centrifuged. If an emulsion occurs at the solvent interface, it is dispersed with a stirring rod and the tube is recentrifuged. The benzene layer is transferred to a similar tube and washed twice by shaking with 5 ml of 0.1 N NaOH. After centrifugation, a 25 ml aliquot of the benzene layer is transferred to a 35 ml tapered glass-stoppered centrifuge tube containing 0.7 ml of 0.1 N HCl. The tube is shaken for 15 minutes and centrifuged, following which the benzene layer is aspirated and discarded. A 0.5 ml aliquot of the acid solution is transferred to a test tube containing 1 ml of 0.13 M borate buffer pH 10. T in this buffered solution is then determined in the spectrophotofluorometer, using 285 mμ for activation and 360 mμ for fluorescence.

A T standard (about 2 μg) and a reagent blank are carried through the entire procedure. The T content of the unknown sample is calculated from the readings obtained with the standard and blank. As little as 0.1 μg of T in 15 ml of urine may be measured by the method. Fluorescence is proportional to T concentrations of 0.1 to 8 μg.

As some drugs may produce extractable fluorescent material which interferes with the determination it is advisable to examine both the activation and fluorescence spectra of the sample (see Fig. 9) when assaying T. The average T excretion in man is about 75 μg per day, with values ranging from 25 to 130 μg.

Norharman fluorescence (HESS and UDENFRIEND 1959, HESS 1961).

The method based on the native fluorescence of T works well with human urine, but is not sensitive and specific enough for the assay of T in tissues or urine of some laboratory animals. A method involving extraction of the T, its condensation with formaldehyde and dehydrogenation to a highly fluorescent product, apparently norharman (see p. 74), exhibits such a great sensitivity as to allow the determination of T present in tissues in concentrations higher than 0.1 μg

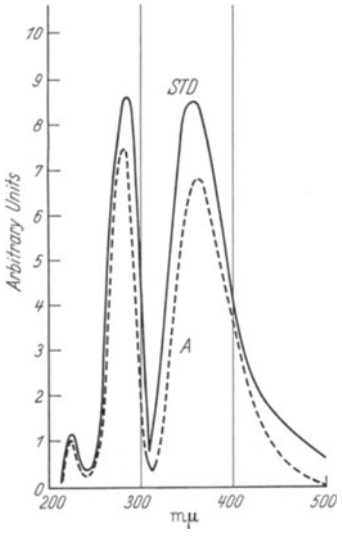


Fig. 9

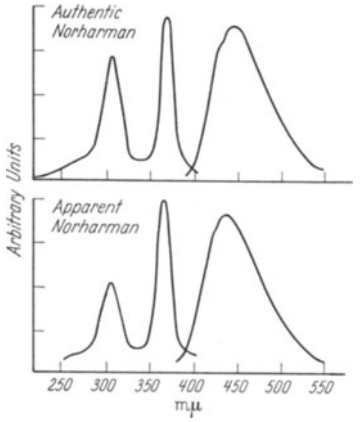


Fig. 10

Fig. 9. Activation and fluorescence spectra of authentic T (STD) and apparent T (A) from urine (SJOERDSMA et al. 1959)

Fig. 10. Activation and fluorescence spectra of authentic and apparent norharman. The apparent norharman was prepared from T extracted from the liver of a guinea pig treated with TP and monoamine oxidase inhibitor (iproniazid). From HESS and UDENFRIEND (1959)

per gram. Since norharman formation only occurs with indoleethylamine derivatives compounds such as IAA and 5-HIAA do not interfere in the determination of T (Fig. 10). However TP gives a comparable fluorophor when treated with formaldehyde and H_2O_2 but it is not extracted by the benzene and therefore does not interfere in the method.

The procedure for tissues is as follows: To 3 ml of tissue homogenate (1 g tissue + 2 ml 0.1 N HCl), or urine containing 0.1 to 5 μg of T, in a glass-stoppered centrifuge tube are added 0.3 ml of 10 N NaOH to bring the pH to about 11. 20 ml of benzene are then added and the tube is shaken for 15 minutes on a shaking machine. The tube is then centrifuged and as much of the benzene as possible is transferred to another tube containing 5 ml of 0.1 N NaOH. The tube is shaken for 5 minutes, centrifuged, and 15 ml of the benzene are transferred to another tube containing 4 ml of 0.1 N H_2SO_4. This tube is also shaken for 5 minutes, centrifuged, and 3 ml of the acid layer transferred to a test tube containing 0.1 ml of 18 per cent formaldehyde. The tube is covered with a glass-marble and placed in a boiling water bath for 20 minutes following which 0.1 ml of 5 per cent aqueous H_2O_2 is added and the heating is continued for another 20 minutes. After cooling to room temperature fluorescence is measured in a spectrophotofluorometer at 440 mμ when activated at 365 mμ.

The distribution of T between the various solvents used in this procedure is such that about 70 to 80 per cent appears in the final extract. Accordingly, standards are prepared by carrying known amounts of T through the entire

procedure. Fluorescence is proportional to T concentration over the entire range. T added to tissues is recovered in 90 to 100 per cent, based on extracted standards.

When the reagents themselves are carried through the entire extraction procedure and compared to 0.1 N H_2SO_4 the resulting fluorescence is equivalent to about 0.2 to 0.3 μg of T. This blank fluorescence limits the sensitivity of the method. The amounts of T normally present in the tissues of several laboratory animals are too small (0.05 to 0.1 μg per gram) to give values significantly above the reagent blank range. The normal values are presumably less than 0.08 μg per gram. However, following the administration of L-TP or of monoamine oxidase inhibitors, the concentration of T in tissues rises to values between 1 and 2 μg per gram (HESS et al. 1959).

c) Other methods for the determination of tryptamine

RODNIGHT (1956) has described a method for the isolation and determination of urinary 5-HT and T. The method, which involves ion-exchange and paper chromatography, is rather time-consuming and not very precise.

Thin-layer chromatography has been applied quantitatively for the assay of T in plasma by EBLE and BROOKER (1962).

2. Tryptophan

a) Colorimetric determination

p-Dimethylaminobenzaldehyde method (UDENFRIEND and PETERSON 1957).

This method, wich is an adaptation of the procedure of GRAHAM et al. (1947) can be used to determine TP in tissues and biological extracts:

Transfer 0.5 ml of the deproteinized sample containing 0.05 to 0.5 μM of TP to a tube containing 2.5 ml of reagent (p-dimethylaminobenzaldehyde, 0.5% in 12 N HCl) and allow to stand for 30 minutes at room temperature, preferably in the dark. Then add 2.5 ml of absolute ethanol and 2 to 3 drops of 0.2% sodium nitrite, and let stand at room temperature for another 30 minutes. The optical density of the resulting blue chromophore is measured at 620 mμ. Standards are prepared at exactly the same time by adding known amounts of TP dissolved in 0.50 ml of solution to 0.5 ml of deproteinized tissue blank and developing the colour described.

In a Beckman spectrophotometer with a 1 cm light path an optical density of about 0.275 is obtained for 0.1 μM of TP. A linear relation between optical density and concentration is obtained up to 0.5 μM of TP.

The sample can be deproteinized by either trichloroacetic acid or zinc acetate and NaOH.

As the p-dimethylaminobenzaldehyde method is not specific for TP the above procedure is not applicable to studies involving the conversion of TP to other indole derivatives (e.g. T or 5-HTP) unless these compounds are first removed.

Xanthydrol method (DICKMAN and CROCKETT 1956).

This method has been elaborated for the determination of TP in plasma and serum but may, with slight modifications, be applicable to other tissues as well:

To 1 ml of plasma in a centrifuge tube are added 5 ml of 1% picric acid and 370 mg of ammonium sulphate. After thorough mixing, the sample is centrifuged for 15 minutes, and the supernatant solution is filtered through a small cotton plug. A 4 ml aliquot is added to a mixture composed of 4 ml of 12 N HCl, 1 ml of glacial acetic acid, and 1 ml of xanthydrol (1 mg per ml in 12 N HCl) in a glass-stoppered tube. The tube is placed in boling water bath for 15 minutes, then cooled in an ice bath. Saturated NaOH is added until the solution reaches pH 3.5 where it becomes colourless. Excess alkali must be avoided or false low results will be obtained. The solution is then extracted with 10 ml of isoamyl acetate and the upper phase is removed. 1.4 ml of 6 N HCl are added to the organic phase and the mixture is shaken in a glass-stoppered test-tube. The aquerous phase is removed and its absorbancy is measured at 510 mμ in a spectrophotometer. The TP content is calculated from a standard curve made up under the same conditions from TP samples of 10 to 80 μg per ml.

b) Spectrophotofluorometric determination

Native fluorescence of TP (DUGGAN and UDENFRIEND 1956).

This method, which is based on the native fluorescence of TP at 360 mμ, when activated at 280 mμ, has been applied to plasma from various laboratory animals and man. TP is the only substance normally present in plasma in concentrations high enough to show a measurable fluorescence under the conditions employed; in man the fasting level is about 10 μg per ml. The method is, however, less suited for other tissues, since they usually contain interfering substances and here TP has to be isolated before its determination can be made.

A 1 ml sample of plasma is diluted with 4 volumes of water and acidified with 0.5 ml of 0.6 N H_2SO_4. Protein is precipitated by the addition of 0.5 ml of 10% sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) with constant stirring and is removed by centrifugation. Tungstate ions, which have a slight quenching effect on TP fluorescence, are precipitated by the addition of 1 ml of 0.25 Mbarium chloride to a 3 ml aliquot of the clear tungstic acid filtrate. The precipitate of barium sulphate and barium tungstate is removed by centrifugation. A 2 ml aliquot of the solution is treated with 0.5 ml of 2 M sodium carbonate to precipitate excess barium ions and bring the pH of the sample to the optimal value for TP fluorescence (pH 11). The barium carbonate precipitate is removed by centrifugation for 10 minutes at 3000 r.p.m. A small portion (1 to 1.5 ml) of the supernatant fluid, which should be clear and colourless, is withdrawn and its fluorescence measured at 360 mμ when activated at 280 mμ in a spectrophotofluorometer. Standards prepared by appropriate dilutions of TP in 0.3 M sodium carbonate as well as internal standards (TP added to plasma) are run together with the unknown samples. A reagent blank in which pure water is substituted for plasma is carried through the entire procedure to serve as a fluorescence blank.

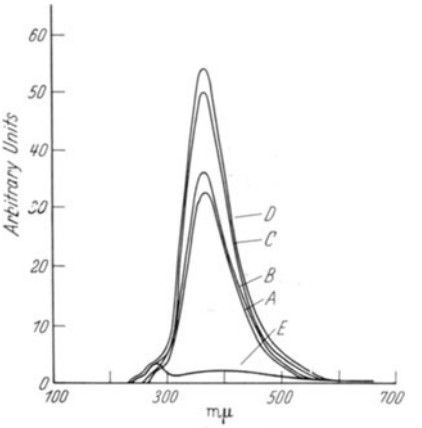


Fig. 11. Fluorescence spectra of TP standards (curves *A* and *C*), plasma TP (curve *B*), plasma plus added TP (curve *D*) and reagent blank (curve *E*). From DUGGAN and UDENFRIEND (1956).

The fluorescence spectra of sample, internal and absolute standards, and blank in a TP determination with the above method is shown in Fig. 11.

Norharman fluorescence (HESS and UDENFRIEND 1959).

When treated with formaldehyde and hydrogen peroxide TP yields — like T — norharman, which is highly fluorescent. This reaction can be applied to trichloroacetic acid filtrates of both plasma and tissue, and offers an extremely specific method for the determination of TP. The sensitivity has been reported to be so great as to allow the assay of TP in fingertip blood. The details of this reaction are given on p. 95, in regard to T.

c) Other methods for the determination of tryptophan

The methods described above are those usually employed in biochemical and pharmacological studies. They are not strictly specific for TP and, of course, do not distinguish between D- and L-TP. The only available procedures which are strictly specific for the L-isomer of TP are those involving microbiological or enzymatic assay. Microbiological assay procedures for L-TP have been published by DUNN et al. (1945) and by SCHWEIGERT et al. (1946). These procedures are, however, rather time-consuming.

SCOTT (1961) has described an enzymatic method for the estimation of L-TP in biological material in the range of 1 to 60 μg. L-TP is converted quantitativly into indole by the enzyme tryptophanase (prepared from acetone dried powder

of Escherichia coli), and the indole measured colorimetrically after extraction with light petroleum, with either p-dimethylaminobenzaldehyde or p-dimethylaminocinnamaldehyde reagents.

3. Indoleacetic acid

IAA can be extracted from acidified solutions into non-polar solvents and then returned to aqueous alkali. This separates IAA from related indoles and other interfering substances and it can then be determined by fluorometric or colorimetric methods.

a) Spectrophotofluorometric determination

(WEISSBACH et al. 1959)

A very sensitive procedure for the determination of IAA is based on its fluorescence at 365 mμ when activated at 285 mμ. The procedure is as follows:

An aliquot of deproteinized sample containing 1 to 10 μg of IAA is transferred to a 40 ml glass-stoppered centrifuge tube and acidified by the addition of 0.3 ml of 6 N HCl. 15 ml of chloroform are added and the tube shaken for 1 to 2 minutes. After centrifugation the aqueous layer is removed and discarded. The chloroform phase is washed by shaking with 10 ml of 0.1 N HCl, thus removing any tryptophan that may have been extracted into the chloroform. 10 ml of the chloroform are then transferred to another 40 ml glass-stoppered shaking tube containing 1.5 ml of 0.5 M phosphate buffer pH 7. After thorough shaking and centrifugation, the chloroform layer is discarded. The aqueous phase is transferred to a cuvette and fluorescence determined in a spectrophotofluorometer (e.g. AMINCO-BOWMAN) at 365 mμ when activated at 285 mμ. Standards and reagent blanks are all treated in the same manner.

b) Colorimetric determination

(WEISSBACH et al. 1959).

This method which is based on the xanthydrol reaction of DICKMAN and CROCKETT (1956) is not as sensitive as the fluorometric procedure but may be used on materials where IAA is present in larger amounts, e.g. in human urine. The procedure is as follows:

4 ml of urine in a 40 ml glass-stoppered centrifuge tube are acidified by the addition of 0.36 ml of 12 N HCl. 10 ml of chloroform are added and the tube shaken for 5 minutes. After centrifugation 9 ml of the chloroform are transferred to another 40 ml glass-stoppered tube containing 0.6 ml of 0.5 M phosphate buffer pH 7. The tube is shaken for 5 minutes and the chloroform layer is aspirated and discarded. An aliquot (0.4 ml) of the buffer extract is transferred to a test tube containing 0.4 ml of 12 N HCl; 1 ml of 0.1% xanthydrol in glacial acetic acid (freshly prepared) is added, followed 5 minutes later by 0.5 ml of 5% sodium bisulfite. The solution is mixed and the pink colour measured within 5 to 10 minutes at 520 mμ in a spectrophotometer. Standards and reagent blanks are treated in the same manner. As little as 8 μg of IAA can be detected by this procedure. Optical density is proportional to IAA concentration over the range from 8 to 200 μg. 20 μg IAA carried through the entire method gives an optical density of 0.130.

IAA is usually excreted in the urine in conjugated form and unless a hydrolysis step is included in the procedure, low values — representing only free IAA — will be obtained. The tubes containing the 4 ml urine sample and 0.36 ml of 12 N HCl (see above) are placed in a boiling water bath for 15 minutes. After cooling, the urine is assayed as described above. The normal urinary excretion of indoleacetic acid in man is 3 to 8 mg (as free IAA) and 5 to 14 mg (as total IAA after acid hydrolysis) per 24 hours.

II. 5-Hydroxyindoles

In pharmacology and biochemistry most studies of the 5-hydroxyindoles concern the active 5-HT, its precursor 5-HTP or its main metabolite 5-HIAA.

In the following section different methods for the quantitative determination of these compounds will be described. Reviews of the detection and estimation of 5-hydroxyindoles and related compounds have been published by UDENFRIEND et al. (1958), DALGLIESH (1958), UDENFRIEND and WEISSBACH (1963) and SANDLER (1963).

Determination of total 5-hydroxyindoles

All 5-hydroxyindoles show the same fluorescence characteristics and in tissues where their total content is greater than 1 μg per gram they may be determined directly after precipitation of the proteins with zinc hydroxide (WEISSBACH et al. 1958, UDENFRIEND and WEISSBACH 1963). The following procedure is recommended:

Tissues are homogenized in at least 2 volumes of 0.1 N HCl, and an aliquot of the homogenate corresponding to no more than 2 grams is made up to 8 ml with water. The proteins are then precipitated by the addition of 1 ml of 10 % zinc sulphate followed by 0.5 ml of 1 N NaOH. After thorough shaking the precipitated protein is removed by centrifugation for 15 minutes at 10.000 $\times$ g. Then 1 ml of the supernatant fluid is transferred to a test tube containing 0.3 ml of 12 N HCl and the fluorescence measured in a spectrophotofluorometer at 550 mμ, using an activation of 295 mμ. Standards (e.g. of 5-HT) and a reagent blank, in which the tissue aliquot is replaced by water, are treated in the same manner.

1. 5-Hydroxytryptamine

The method from which most others for 5-HT assay derive to a greater or lesser extent, is that of UDENFRIEND et al. (1955b), the principle of which was described above in the discussion of extraction procedures (see p. 67).

5-Hydroxytryptamine in tissues

The extraction method which will be described below can be used for most tissues, e.g. brain, lung, gastric and intestinal mucosa, spleen, skin and tumour material (carcinoids). However, for blood and plasma of most animal species this method is not sufficiently sensitive and alternate methods must be employed (see p. 101).

Reagents. Borate Buffer. To 94.2 g of boric acid in 3 liters of water are added 165 ml of 10 N NaOH. The buffer solution is then saturated with purified n-butanol and NaCl by adding these substances in excess and shaking. Excess n-butanol is removed by aspiration and excess salt permitted to settle. The final pH should be approximately 10.

n-Butanol. Reagent grade n-butanol is purified by shaking first whith an equal volume of 0.1 N NaOH, then with an equal volume of 0.1 N HCl, and finally twice with distilled water.

Heptane. Practical grade of heptane is treated in the same manner as the n-butanol.

Procedure. The tissues are homogenized in 2 parts of 0.1 N HCl. A 3 ml aliquot of the homogenate is transferred to a 60 ml glass-stoppered bottle and adjusted to pH 10 by the addition of anhydrous sodium carbonate. After addition of 5 ml of borate buffer pH 10 the solution is diluted with water to a volume of 15 ml and 5 g of NaCl and 15 ml of n-butanol are added. The bottle is shaken for 10 minutes, then centrifuged and the fluid decanted from the solid material into a glass-stoppered centrifuge tube. After centrifugation the aqueous layer is removed by aspiration and the butanol phase washed by shaking with an equal volume of borate buffer. After centrifugation 10 ml of the butanol phase is transferred to another tube containing 20 ml of heptane and 1.5 ml of 0.1 N HCl. After shaking and centrifugation of the tube the supernatant solvent is removed. The aqueous phase containing the extracted 5-HT is then assayed by one of the three following methods:

α) Assay by ultraviolet absorption. The absorption of 5-HT at 275 mμ can be used for the assay when the amine is present in amounts of 0.08 to 0.8 μM per gram of tissue (see p. 73). The method can be applied to studies of the activities of 5-HTP decarboxylase and monoamine oxidase in tissue preparations. The amounts of 5-HT involved in such studies are usually large and the extraction procedure also effectively separates it from either precursor (5-HTP) or end-

product (5-HIAA). In experiments where 5-HT is present in tissues, the butanol extract should be washed three times with borate buffer instead of once.

β) Colorimetric assay. The 1-nitroso-2-naphtol method (see p. 79 for procedure) has about the same sensitivity as the ultraviolet absorption technique. However, it is the method of choice when tissue extracts contain large amounts of ultraviolet-absorbing material.

γ) Spectrophotofluorometric assay. The extreme sensitivity of the spectrophotofluorometric assay offers a distinct advantage over both the ultraviolet absorption and the colorimetric method. Depending on the nature of the material to be studied, two methods of measuring the fluorescence may be used:

a) Fluorescence at 330 mμ. In weakly acid solutions (pH 2—4) 5-HT shows a maximal fluorescence at 330 mμ when activated at 295 mμ. This is the most sensitive method available for 5-HT assay. In pure solutions 0.01 μg per ml may be detected.

When this technique is used the final acid solution in the extraction procedure should be adjusted to pH 2—4 — or the 0.1 N HCl replaced by a 0.5 M formate buffer of pH 4 — to obtain maximal fluorescence.

b) Fluorescence at 550 mμ. Extracts of certain tissue (e.g. brain) normally contain materials that fluoresce near 330 mμ and therefore make analysis by the above technique difficult. In such cases the fluorescence at 550 mμ shown by 5-HT in strong acid (3 N HCl), when activated at 295 mμ, is the method of choice. Under these conditions very little interference from other physiological substances occurs. The fluorescence at 550 mμ, although less sensitive than the 335 mμ fluorescence at pH 2—4, is thus more specific for 5-HT and is now the method of assay generally employed in studies of biological material. As little as 0.1 μg of 5-HT can be measured by this technique.

When using the 550 mμ fluorescence for assay, 1 ml of the final HCl extract is added to 0.3 ml of concentrated HCl in a quartz cuvette and the fluorescence determined in a spectrophotofluorometer with activation at 295 mμ (for further details see p. 74).

Specificity of the extraction method

The specificity of the technique described above for the assay of 5-HT has been thoroughly discussed by BOGDANSKI et al. (1956) and UDENFRIEND et al. (1958). The material extracted from brain exhibit the same activation and fluorescence spectra as 5-HT. In a counter-current extraction system the 550 mμ fluorescing material was distributed almost identically with 5-HT (see p. 81), and also had the same pharmacological activity on the *Venus mercenaria* heart. Material isolated from rabbit stomach and intestine also proved to be chromatographically identical with 5-HT.

Nevertheless, the technique is not absolutely specific for 5-HT since N-methyl-5-HT and N,N-dimethyl-5-HT, (bufotenine) are also extracted to a minor degree. Fortunately, these compounds are not normally present in mammalian tissues. 5-Methoxy-T, which has the same fluorescence spectrum as 5-HT, also interferes with the procedure. However, 5-methoxyindoles seem thus far to be limited in occurrence to the pineal gland in mammalia, and here occur in rather minute amounts.

Sensitivity and recovery

5-HT added to brain was recovered to the extent of 92—102%. Approximately 0.1 μg of 5-HT can be detected in the procedure but at least 0.3 μg are needed for accurate assay (BOGDANSKI et al. 1956).

Modifications of the extraction method

A number of modifications of the Udenfriend extraction method have been reported. However most of these concern minor details and seem to have resulted in little improvement of the original technique. Accordingly only a few of these modifications will be mentioned.

DALGLIESH and DUTTON (1957) have reported that better recoveries are obtained with a borate buffer of pH 9.5—9.8 than with of pH 10. CRAWFORD and RUDD (1962) confirmed this observation. In the method of DALGLIESH and DUTTON the final extraction of 5-HT is further made into 3 N HCl instead of dilute HCl and its fluorescence measured in a filter fluorometer. The method is reported to be satisfactory to a concentration of 0.02 μg per ml of 5-HT.

In the method of CRAWFORD and RUDD (1962) proteins are precipitated with trichloroacetic acid, which is then removed by ether extraction. The rest of their method differs only in the use of isooctane instead of heptane, presumably used because pure isooctane is available commercially.

CAIS (1961) deproteinizes sample with perchloric acid and extracts with butanol at pH 11; he replaces heptane with petroleum ether.

5-Hydroxytryptamine in blood

α) Direct assay after protein precipitation. In some animals (e.g. rabbits and chickens) as well as in patients with carcinoid tumours, 5-HT occurs in blood in sufficiently high concentrations (i.e. above 1 μg per ml) to permit an assay by fluorescence techniques made directly on the deproteinized blood filtrate. The procedure, which is that of WEISSBACH et al. (1958), is as follows:

The blood to be assayed is collected in a siliconized vessel containing anticoagulant (heparin). 1 ml of blood is diluted with 6 ml of water, deproteinized with zinc sulphate and sodium hydroxide and then further treated as described above for the determination of total 5-hydroxyindoles (p. 99).

5-HT concentrations exceeding 0.5—1 μg per ml of blood can be measured by this method . The recovery of 5-HT added to blood is from 85 to 95%.

It must be kept in mind that this is a method for *total 5-hydroxyindoles* measuring not only 5-HT but all other indoles hydroxylated in the 5 position which may be present in the blood. In rabbits and chickens, 5-HT is the only 5-hydroxyindole present, and here the method may be considered specific for this compound.

However, in patients which carcinoid tumours, 5-HIAA is occasionally found in the blood in concentrations as high as 0.5 to 1 μg per ml. In such cases 5-HT must be separated from this compound, and possibly from other 5-hydroxyindoles by extraction of the deproteinized blood with butanol (see below).

β) Assay after protein precipitation and butanol extraction. The 5-HT concentration in blood of many species (e.g. man, dog, guinea-pig, rat and mouse) is so low (less than 0.5 μg per ml) that the above direct method cannot be used for its assay. A combined protein precipitation and butanol extraction procedure has proved satisfactory, however. Such a procedure not only results in specificity, but also increases the sensitivity of the method, since a large volume of deproteinized blood filtrate can be extracted, and since the 5-HT is returned to a small volume of dilute acid.

WAALKES (1959) has described a sensitive fluorometric method for 5-HT in human blood and plasma which is based on the above combination of protein precipitation and butanol extraction. A 3 ml sample of whole blood or plasma collected with EDTA as anticoagulant, is deproteinized with zinc hydroxide. After salt saturation of the blood filtrate, the 5-HT is isolated and concentrated

by two alternative methods; either the ordinary butanolheptane method or the ethyl acetate extraction followed by cotton acid succinate as an ion exchange medium. In both methods the 5-HT is obtained in an acid solution, and it is determined by its fluorescence at 550 mμ when activated at 295 mμ.

The range of values for the 5-HT in human blood was found by this method to be 0.09 to 0.18 μg per ml.

5-Hydroxytryptamine in blood platelets

Virtually all the circulating 5-HT found in blood is contained in the platelets.

The method of DILLARD et al. (1951) has been recommended for the isolation of platelets (WEISSBACH et al. 1958, UDENFRIEND et al. 1958, UDENFRIEND and WEISSBACH 1963). The platelets obtained by this method from a 10 ml blood sample are suspended in a measured volume (about 3.5 ml) of saline. An aliquot is taken for protein determination and the remainder used for spectrophotofluorometric 5-HT assay, either using the direct method (see p. 99) or the butanol extraction method (see p. 99). The results are expressed as μg of 5-HT per mg of platelet protein.

5-HT seems to be the only 5-hydroxyindole present in platelets. The direct method of determination is therefore well suited for platelet studies provided the 5-HT concentration is not too low for detection.

5-Hydroxytryptamine in urine

Only small amounts of unchanged 5-HT are normally excreted in the urine (e.g. in humans 50 to 100 μg per day). However, its determination may be indicated on various occasions; in studies of amino acid decarboxylase inhibitors when 5-HTP has been administered or in patients with the carcinoid syndrome. In the latter the 5-HT excretion may increase to 1—2 mg per day.

5-HT in urine may be determined by the method of UDENFRIEND et al. (1955b), described above, but a more recent method of OATES (1961) is the one of choice because of its sensitivity, high recovery and comparative simplicity. The procedure, which employs a carboxylic acid ion exchange resin to separate 5-HT from the urine and a spectrophotofluorometric assay of the latter in the eluate, is as follows:

Amberlite IRC-50 resin (H^+ form) is converted to the NH^+ form by washing twice with approximately 3 volumes of 3 N NH_4OH. The resin is then washed three times with 3 volumes of water and stored in 0.2 N ammonium acetate at pH 7.5.

In a 20 cm long ion exchange column is placed a 50×5 mm layer of Amberlite IRC-50 prepared as above. The resin is washed on the column with 5 ml of 0.2 N ammonium acetate pH 7.5 A 1 to 5 ml sample (the volume depending on the expected 5-HT concentration) of the filtered urine is diluted to 6 ml with water. The sample is then adjusted to pH 7.5 with 0.2 N NaOH and applied to the column. After the sample has passed through, the column is carefully washed, first with 10 ml and then 5 ml of 0.02 N ammonium acetate pH 7.5. The 5-HT is then eluted from the column with 5.5 ml of 1 N HCl and the eluate is collected in a graduated tube and made up to 6 ml with 0.1 N HCl. A 1 ml aliquot of the eluate is transferred to a small tube containing 0.3 ml of 12 N HCl and the fluorescence at 540 mμ measured in a spectrophotofluorometer when activated at 290 mμ. Standards which contain 10 μg of 5-HT in 6 ml water, and a water blank, are carried through the procedure together with the unknown sample. The fluorescence is proportional to the 5-HT concentration up to 20 μg in the sample.

Recovery of 5-HT added to urine is 95±5 %. The lower limit of sensitivity of the method is approximately 125 μg of 5-HT per liter of urine when a 5 ml sample is applied to the column.

Urine for 5-HT assay should be kept refrigerated during the collection period and frozen if not analyzed immediately.

Other Methods for the assay of 5-hydroxytryptamine

MEAD and FINGER (1961) have used the catecholamine extraction method of SHORE and OLIN (1958) and found that 5-HT passes into acid butanol as long as salt is present. This renders possible a determination of both the catecholamines and 5-HT on the same tissue homogenate . The acid extraction method is not satisfactory if 5-HTP is present. However, WIEGAND and SCHERFLING (1962) overcame this problem by passing the acid extract from heptane thorough a cation exchange resin with separate elution of 5-HTP, followed by 5-HT.

Several methods employing only ion exchange columns for the separation of 5-HT have been reported. DAVIS and AWAPARA (1960) in their studies of amino acid decarboxylases separated 5-HT from 5-HTP by using a weak cation exchange resin, Amberlite CG-50-H^+. The amino acid passes through the column and the 5-HT is retained, and subsequently eluted with a weak acid.

GLUCKMAN and ABRAMS (1959) employed two columns, and their method is briefly as follows: Tissue homogenates are made with two parts of 0.5 perchloric acid. Following centrifugation, the precipitate is washed twice by rehomogenization with 0.5 M perchloric acid. The combined supernatants are passed through a Dowex 1-OH^- column to remove the perchlorate ions. The eluate is neutralized to pH 7 and its volume reduced to half in a flash evaporator. This material is passed through an Amberlite XE-97-NH_4^+ column. The column is washed with an equal volume of 0.03 M ammonium acetate buffer pH 7 and the 5-HT finally eluted with 1 M formic acid and determined spectrophotofluorometrically (fluorescence 540 mμ and activation 295 mμ).

Several workers have used paper chromatographic and paper electrophoretic procedures for the assay of 5-HT. Some of these methods have been reviewed in the above section on paper chromatography and electrophoresis (see p. 89 and 94). However, most of them are rather laborious, not very accurate and derive from an earlier period of indole biochemistry and pharmacology.

2. 5-Hydroxytryptophan

The amounts of 5-HTP normally present in animal tissues are too small to be detected by any of the methods presently available. However, in experimental work where this amino acid is administered to animals, large amounts (5 μg per gram tissue) are found in the tissues and may be determined spectrophotofluorometrically (UDENFRIEND et al. 1957a). The following procedure is recommended for this type of work.

Spectrophotofluorometric determination (UDENFRIEND et al. 1957a).

To determine 5-HTP the tissues are homogenized in 2 volumes of 0.1 N HCl. 1 ml of this homogenate is diluted with 2 ml of water, and is deproteinized by the addition of 1 ml of 40% trichloroacetic acid. After centrifugation, a 3 ml portion of the supernatant solution is extracted twice with ether to remove trichloroacetic acid and any 5-HIAA present. After the addition of 0.5 ml of 20% Na_2CO_3 to the aqueous phase, it is extracted with 15 ml of n-butanol to remove 5-HT. To 1 ml of the residual aquous solution is added 0.6 ml of 12 N HCl and the fluorescence at 550 mμ measured in a spectrophotofluorometer, using 295 mμ as activation wavelength. Standards of known amounts of 5-HTP are carried through the entire precipitation and the extraction procedure. Reagent blanks are run in the same manner, water being substituted for tissue homogenate.

Tissues from normal animals contain no detectable amounts of 5-HTP. However, they do contain small amounts of some material showing a nonspecific fluorescence at 550 mμ. This fluorescence should be substracted from the values obtained after 5-HTP administration.

When the above method is used it is always advisable to check its specificity by paper chromatography of the residual aqueos extract.

Other methods for 5-hydroxytryptophan

SANDLER and SNOW (1958) have described a small group of carcinoid patients having certain atypical chemical features. In these patients, the urine contains not only 5-HIAA but also relatively large quantities of both 5-HTP and 5-HT. It has been suggested that in these cases the main tumour product is not 5-HT but 5-HTP some of which is then decarboxylated to 5-HT elsewhere in the body (SANDLER et al. 1961).

After Sandlers report the determination of this amino acid in urine of carcinoid patients has assumed increasing importance. SJOERDSMA et al. (1960) have elaborated a method for the determination of 5-HTP in urine which may also be used successfully on tissue extracts. After adjusting to pH 8.4 diluted urine is passed through an Amberlite IRC 50 column which removes any 5-HT present. The column effluent is incubated with a guinea-pig decarboxylase preparation, made from animals which have been pretreated with a monoamine oxidase-inhibitor so that 5-HT formed during incubation is not further metabolized. The incubation mixture is then passed through another IRC 50 column, from which the 5-HT is eluted and measured spectrophotofluorometrically.

3. 5-Hydroxyindoleacetic acid

5-Hydroxyindoleacetic acid in urine

The greater part of 5-HT formed in the body is excreted as 5-HIAA which is a normal constituent of mammalian urine (TITUS and UDENFRIEND 1954, ERSPAMER 1955). Measurement of the urinary excretion of 5-HIAA can, therefore, be used as an indication of the production and metabolism of 5-HT in the body.

Either ultraviolet absorption or fluorescence can be used for determining 5-HIAA, just as for 5-HT as has been already described. However, these methods are less readily applicable to urine than in the case of 5-HT, as extracts containing 5-HIAA essentially free of interfering substances are less readily obtained from urine than are extracts containing 5-HT.

Colorimetric determination.

Colorimetric procedures which are less influenced by other substances in the urine extracts are usually applied for the determination of 5-HIAA. Most procedures described are based either on the 1-nitroso-2-naphtol or the p-dimethylaminobenzaldehyde reaction (see p. 79 and 77).

1-Nitroso-2-naphtol methods

The most popular procedure seems to be a 1-nitroso-2-naphtod methol introduced by UDENFRIEND et al. (1955c). The method depends on the ability to extract 5-HIAA from a salt-saturated aqueous solution into ether. Keto acids, which interfere with the colour reaction, are removed from the urine by treatment with 2,4-dinitrophenylhydrazine and IAA which gives a slight colour with nitrous acid, is extraced with chloroform.

Reagents. *1-Nitroso-2-naphtol*, 0.1% solution in ethanol.

Nitrous acid reagent. To 5 ml of 2 N H_2SO_4 is added 0.2 ml of 2.5% $NaNO_2$. This reagent should be prepared fresh daily.

Ethyl ether. Reagent grade washed once with a dilute solution of ferrous sulphate and twice with water, to remove peroxides.

2,4-Dinitrophenylhadrazine, 0.5% in 2 N HCl.

Phosphate buffer. 0.5 M, pH 7.

Procedure. To 6 ml of urine are added 6 ml of 2,4-dinitrophenylhydrazine solution. After 30 minutes, 25 ml of chloroform are added and the mixture shaken for a few minutes and

then centrifuged. The organic layer is removed, replaced with a fresh 25 ml portion of chloroform and the extraction repeated. After centrifugation, a 10 ml aliquot of the aqueous layer is transferred to a glass-stoppered centrifuge tube containing about 4 g of NaCl and 25 ml of ether. The mixture is shaken for 5 minutes. After centrifugation, a 20 ml aliquot of the ether is transferred to another glass-stoppered centrifuge tube containing 1.5 ml of phosphate buffer. The tube is shaken for 5 minutes, centrifuged, and the ether layer is removed by aspiration. 1 ml of the aqueous phase is transferred to a small glass-stoppered centrifuge tube containing 0.5 ml of 1-nitroso-2-naphtol reagent. Following this, 0.5 ml of nitrous acid reagent is added. The sample is then mixed well and warmed at 37° for 5 minutes. Unreacted 1-nitroso-2-naphtol is removed by extraction of the sample with two 5 ml portions of ethyl acetate. The optical density of the final coloured aqueous layer is measured at 540 mμ. Standards of 10 to 200 μg of 5-HIAA per 6 ml and a reagent blank of 6 ml water are treated in the same manner as the unknown sample.

The recovery of 5-HIAA added to urine is about 85%.

The range of urinary 5-HIAA excretion in normal man was found by this method to be 2.0 to 8.2 mg per 24 hours; in dogs, 1.4 to 2.7 mg; and in rabbits, 0.3 to 0.6 mg per 24 hours.

Other methods based on the 1-nitroso-2-naphtol reaction

The above method of UDENFRIEND et al. (1955c) is rather laborious and low recoveries have been reported by several workers. Modifications have therefore been made. Rivalling it in popularity is the method of MACFARLANE et al. (1956; see also DALGLIESH 1958). This method is shorter; requiring about one half the time when compared with the original method. The 2,4-dinitrophenylhydrazine step is omitted, as urines containing significant amounts of keto acids are considered, *a priori*, to be rare. The chloroform step is also omitted, as the interfering pink colour with IAA is quantitatively extracted at the ethyl acetate step of the colour reaction. As all extractions are done in acid solution in which 5-HIAA is relatively stable, it is unneccessary to use peroxide-free ether to prevent destruction of 5-HIAA. The procedure is as follows:

To 5 ml of urine, adjusted to pH 3 with acetic acid, are added 2 g of NaCl and 25 ml of ether. The mixture is shaken for 1 minute and the two phases are then separated by centrifugation. 20 ml of the ether layer is transferred to a flask in which the ether is rapidly removed under reduced pressure. The residue is taken up in 4 ml of water and a measured volume (up to 3 ml, the volume depending on the 5-HIAA content) is transferred to a test tube. 1 ml each of 1-nitroso-2-naphtol reagent and nitrite reagents (see above) are added, and the tubes warmed at 55° for 5 minutes. The solution is then extracted with 10 ml of ethyl acetate, which removes both excess 1-nitroso-2-naphtol and the interfering colour given by IAA. The optical density of the lower layer is read at 540 mμ, preferably after half an hour. The results are obtained from a standard curve prepared at the same time with pure 5-HIAA in amounts of 0 to 90 μg. Recoveries are essentially quantitative, and the standard curve can be made without putting the pure 5-HIAA through the entire procedure. A standard curve may also be prepared using equivalent amounts (on a 5-hydroxyindole basis) of the more stable 5-HT.

The mean excretion of 5-HIAA of man was found to be 7 mg per 24 hours with a range of 3.2 to 13.7 mg.

SCHÖN et al. (1960) obtained very low recoveries (25 to 56%) when 5-HIAA was added to urine and determined by the original method of UDENFRIEND et al. (1955c). They postulated that this was due to losses incurred at the chloroform extraction step due to formation of emulsions during prolonged shaking. They therefore omitted the chloroform step and passed a dried ethyl acetate extract through an alumina column before taking it back into buffer for the colour reaction with 1-nitroso-2-naphtol.

Drugs interfering with the 1-nitroso-2-naphtol reaction

A source of error in the determination of 5-HIAA in urine arises from the ingestion of certain drugs. After ingestion of phenacetin and related drugs,

p-hydroxyacetanilide occurs in the urine and gives a colour reaction (UDENFRIEND et al. 1955b). Ingestion of mephenesin, 3-ortho-toloxy-1,2-propanediol (CURZON 1955, HORNET et al. 1959) and methocarbamol, 3-(o-methoxyphenoxy)-1,2-propanediol monoacarbamate (HORNET et al. 1959) results in metabolites which give an unspecific colour reaction of the urine with 1-nitroso-2-naphtol in the chemical carcinoid screening test of SJOERDSMA et al. (1955). Glyceryl ether of guaiacol, which is widely used as expectorant, also produces urinary metabolites which interfere with the 1-nitroso-2-naphtol reaction — not only in the screening test (SJOERDSMA 1958) but also in the quantitative method of UDENFRIEND et al. (1955c). After ingestion of therapeutic doses of this drug the determination shows falsely high values for 5-HIAA, as high as those usually encountered in malignant carcinoid (PITKÄNEN et al. 1962). Some metabolic product of glyceryl guaiacol ether seems to be the cause of the reaction, as the drug does not react in vitro.

Various derivatives of phenothiazine (e.g. chlorpromazine) have been found to cause a quenching of the colour in the 1-nitroso-2-naphtol reaction (SJOERDSMA et al. 1957, ROSS et al. 1958). CHADWICK and WILKINSON (1960) have made a careful study of this quenching effect and found that although these drugs markedly interfere with the screening test of SJOERDSMA et al. (1955), their effect can be minimized by using the more specific extraction method of UDENFRIEND et al. (1955c) before the colour reaction.

p-Dimethylaminobenzaldehyde methods

The p-dimethylaminobenzaldehyde reaction (see p. 77) has been used by several workers for the determination of 5-HIAA in urine.

HANSON and SERIN (1955) used the following procedure for studies of the 5-HIAA excretion in patients with malignant carcinoid:

A 2 ml aliquot of the filtered urine is acidified with one or two drops of 10% HCl and twice extracted with 25 ml of ether. The pooled ether extracts are then filtered, dehydrated with about 3 g anhydrous sodium sulphate, and evaporated to dryness at 50°. The residue is dissolved in 1 ml of 0.1 N HCl, and to 0.2 ml of this solution in a test tube is added 5 ml of p-dimethylaminobenzaldehyde reagent (2% solution in 20% HCl). The solution, and a blank consisting of 0.2 ml of 0.1 N NCl and 5 ml of reagent, are then heated for 2 hours in a water bath at 45—50°. The solutions are next removed from the water bath and immediately diluted with cold 50% ethanol to a volume of 10 ml and the optical density measured in a spectrophotometer at 590 mμ. The results are read from a standard curve made up from known amounts of 5-HIAA. This method permits a determination of 5 μg of 5-HIAA (i.e. 12.5 mg per 1000 ml of urine). The method is not specific for 5-HIAA (see p. 77). However, in cases of malignant carcinoid the 5-HIAA is always the predominating indole and the method is satisfactory for routine clinical work.

ERSPAMER (1956) has used the p-dimethylaminobenzaldehyde reaction after paper chromatographic separation. Concentrated urine is repeatedly precipitated with alcohol-acetone and acetone-ethyl ether (ERPSAMER 1955, see also p. 67) and then chromatographed on paper using the n-butanol-acetic acid-water mixture (4:1:5) as solvent and p-dimethylaminobenzaldehyde reagent as locating reagent. The 5-HIAA spots are then visually compared with respect to area and density of colour with spots obtained with known amounts of pure 5-HIAA.

Another, more precise paper chromatographic method is based on the elution of the spots with water and colorimetric determination of 5-HIAA in the eluate with p-dimethylaminobenzaldehyde reagent (ERSPAMER 1955). To 2 volumes of the eluate is added 1 volume of freshly prepared 2% solution of p-dimethylaminobenzaldehyde in concentrated HCl. The solution is then placed in a water bath at 56—58° for 10—12 hours. The coloured solution is then read in a spectrophotometer at 600 mμ (CORREALE 1955).

In experiments in which 5-HIAA was added to fresh human and dog urine recovery was between 90 and 100% with these paper chromatographic methods.

Storage of urine for 5-hydroxyindoleacetic acid assay

In animal experiments, as well as in clinical work, urine samples for 5-HIAA assay often have to be stored for later analysis. A consensus seems to be that the most satisfactory way to obtain minimal destruction is to bring the urine immediately to pH 2—3 with HCl (DEGKWITZ et al. 1962, FUCHS and FUCHS 1960). When stored for longer periods the acidified samples should be kept frozen.

Fruit diet and 5-hydroxyindoleacetic acid assay

A number of varieties of fruit have been found to contain relatively large amounts of 5-HT. Walnuts may contain as much as 170—340 μg per gram (KIRBERGER and BRAUN 1961), banana peels 50—150 μg per gram and banana pulp 20—30 μg per gram (ANDERSON et al. 1958, UDENFRIEND et al. 1959), grape fruit 10—20 μg per gram (BRUCE 1960, WEST 1960) and tomatoes 5—6 μg per gram (WEST 1958). After ingestion of these fruits 5-HT is metabolized to and excreted as 5-HIAA which may result in falsely high values when endogenous excretion is determined. Ingestion of walnuts and bananas may even increase 5-HIAA excretion enough to falsely suggest a diagnosis of malignant carcinoid (see e.g. CROUT and SJOERDSMA 1959, KIRBERGER 1962).

In experimental studies on 5-hydroxyindole metabolism, as well as in clinical work, it is therefore absolutely mandatory to omit these fruits from the diet before specimens of urine are collected for assay.

5-Hydroxyindoleacetic acid in tissues

The amounts of 5-HIAA normally found in tissues are too small to be determined. However, when 5-HT is added to crude tissue preparations which contain monoamine oxidase and aldehyde dehydrogenase it is oxidized to 5-HIAA via 5-hydroxyindoleacetaldehyde. In studies of this reaction 5-HIAA can be determined spectrophotofluorometrically by the following procedure (UDENFRIEND and WEISSBACH 1963):

An aliquot of the incubation mixture (containing about 2 μg of 5-HIAA is transferred to a glass-stoppered tube, acidified with 1 ml of 1 N HCl, and diluted to 4 ml. The aqueous phase is saturated with solid NaCl, and extracted with 15 ml of peroxide-free ether. After centrifugation, 10 ml of the ether layer are transferred to another tube containing 1.5 ml of 0.5 M phosphate buffer pH 7. After shaking and centrifuging the buffer layer is assayed spectrophotofluorometrically at 340 mμ, when activated at 295 mμ. Standards and reagent blanks are treated in the same manner.

References

ANDERSON, J. A., M. R. ZIEGLER, and D. DOEDEN: Banana feeding and urinary excretion of 5-hydroxyindoleacetic acid. Science **127**, 236—238 (1958).

ARMSTRONG, M. D., K. N. F. SHAW, M. J. GORTATOWSKI, and H. SINGER: The indole acids of human urine. Paper chromatography of indole acids. J. biol. Chem. **232**, 17—30 (1958).

— — and P. E. WALL: The phenolic acids of human urine. J. biol. Chem. **218**, 293—303 (1956).

AXELROD, J., R. O. BRADY, B. WITKOP, and E. V. EVARTS: The distribution and metabolism of lysergic acid diethylamide. Ann. N.Y. Acad. Sci. **66**, 444—435 (1957).

—, and H. WEISSBACH: Purification and properties of hydroxyindole-O-methyl transferase. J. biol. Chem. **236**, 211—213 (1961).

BLAU, K.: Chromatographic methods for the study of amines from biological material. Biochem. J. **80**, 193—200 (1961).
BLOCK, R. J., E. L. DURRUM, and G. ZWEIG: A manual of paper chromatography and paper electrophoresis. New York 1958.
BOARDMAN, N. K.: Molecular sieving other than dialysis. In: H. F. LINSKENS and M. V. TRACEY (eds.), Modern methods of plant analysis, vol. 5, p. 205—213. Berlin-Göttingen-Heidelberg: Springer 1962.
BOBBIT, J. M.: Thin-layer chromatography. New York 1963.
BOGDANSKI, D. F., A. PLETSCHER, B. B. BRODIE, and S. UDENFRIEND: Identification and assay of serotonin in brain. J. Pharmacol. exp. Ther. **117**, 82—88 (1956).
BOWMAN, R. L., P. A. CAULFIELD, and S. UDENFRIEND: Spectrophotofluorometric assay in the visible and ultraviolet. Science **122**, 32—33 (1955).
BRUCE, D. W.: Serotonin in pineapple. Nature (Lond.) **188**, 147 (1960).
BUMPUS, F. M., and I. H. PAGE: Serotonin and its methylated derivatives in human urine. J. biol. Chem. **212**, 111—116 (1955).
BURCHFIELD, H. P., and E. E. STORRS: Biochemical applications of gas chromatography. New York 1962.
CAIS, M.: The spectrofluorometric assay of serotonin and histamine in tissues following a common extraction procedure. Med. exp. (Basel) **5**, 73—76 (1961).
CARCASONA, A., J. CERVOS-NAVARRO u. F. UNTERHARNSCHEIDT: Untersuchungen zur quantitativen Bestimmung von 5-Hydroxytryptamin und 5-Hydroxyindolessigsäure mit dem Spektralphotometer. Klin. Wschr. **37**, 253—254 (1959a).
— F. UNTERHARNSCHEIDT u. J. CERVOS-NAVARRO: Die quantitative Bestimmung von 5-Hydroxytryptophan, 5-Hydroxytryptamin und 5-Hydroxyindolessigsäure mit der Papierchromatographie. Klin. Wschr. **37**, 761—763 (1959b).
— — — u. W. GELLER: Untersuchungen zur quantitativen Bestimmung von 5-Hydroxytryptophan, 5-Hydroxytryptamin und 5-Hydroxyindolessigsäure mit Hilfe der Papierelektrophorese. Klin. Wschr. **38**, 457—460 (1960).
CHADWICK, B. T., and J. H. WILKINSON: The effects of phenothiazine drugs on the determination of urinary 5-hydroxyindolylacetic acid. Clin. chim. Acta **5**, 879—886 (1960).
CHERONIS, N. D., and J. B. ENTRIKIN: Semimicro qualitative organic analysis, p. 730. New York 1957.
CLOTTEN, R., u. A. CLOTTEN: Hochspannungs-Elektrophorese. Stuttgart 1962.
CONSDEN, R.: Paper chromatography. In C. LONG (ed.), Biochemists' Handbook, p. 127—160. London 1961.
COOPER, J. R., and I. MELCER: The enzymic oxidation of tryptophan to 5-hydroxytryptophan in the biosynthesis of serotonin. J. Pharmacol. exp. Ther. **132**, 265—268 (1961).
CORREALE, P.: Colorimetric and spectrophotometric titration of 5-hydroxyindoleacetic acid. Experientia (Basel) **11**, 315 (1955).
CRAIG, L. C.: Countercurrent distribution. In: C. LONG (ed.), Biochemists' Handbook, p. 98—105. London 1961.
—, and D. CRAIG: Extraction and distribution. In: A. WEISSBERGER (ed.), Technique of organic chemistry, vol. 3, p. 171—311. New York 1956.
CRAWFORD, N., and B. T. RUDD: A spectrophotofluorometric method for the determination of serotonin (5-hydroxytryptamine) in plasma. Clin. chim. Acta **7**, 114—121 (1962).
CROUT, J. R., and A. SJOERDSMA: The clinical and laboratory significance of serotonin and catecholamines in bananas. New Engl. J. Med. **261**, 23—26 (1959).
CURZON, G.: A rapid chromatographic test for high urinary 5-hydroxy-indole-acetic acid and 5-hydroxytryptamine. Lancet **1955 II**, 1361—1362.
DALGLIESH, C. E.: Isolation and examination of urinary metabolites containing an aromatic system. J. clin. Path. **8**, 73—78 (1955).
— Two-dimensional paper chromatography of urinary indoles and related substances. Biochem. J. **64**, 481—485 (1956).
— The 5-hydroxyindoles. Advance. clin. Chem. **1**, 193—235 (1958).
—, and R. W. DUTTON: Biogenesis of 5-hydroxytryptophan. Brit. J. Cancer **11**, 296—309 (1957).
DAVIS, V. E., and J. AWAPARA: A method for the determination of some amino acid decarboxylases. J. biol. Chem. **235**, 124—127 (1960).
DEAVER, G. L., W. E. CRISP, and W. J. FRAJOLA: Use of ionophoretic mobility equilibria in fractionation of mixtures containing indoles. Proc. Soc. exp. Biol. (N.Y.) **98**, 632—635 (1958).
DEGKWITZ, R., R. FROWEIN, E. KIRBERGER, C. KULENKAMPFF u. U. MOHS: Über Normalwerte der stündlichen 5-HIES-Ausscheidung im Urin beim Menschen und die Messungen störende Faktoren. Klin. Wschr. **40**, 285—289 (1962).
DEMOLE, E.: Chromatostrips and chromatoplates. Chromatogr. Rev. **1**, 1—10 (1959).

DENFFER, D. VON, M. BEHRENS u. A. FISCHER: Papierelektrophoretische Trennung von Indolderivaten aus Pflanzenextrakten. Naturwissenschaften **39**, 258—259 (1952).
DENGLER, H.: Atypisches Carcinoidsyndrom mit vermehrter Ausscheidung von 5-Hydroxyindolessigsäure bei Pankreascarcinom. Klin. Wschr. **37**, 1245—1248 (1959).
DICKMAN, S. R., and A. L. CROCKETT: Reactions of xanthydrol. IV. Determination of tryptophan in blood plasma and in proteins. J. biol. Chem. **220**, 957—965 (1956).
—, and W. L. WESCOTT: Reactions of xanthydrol. I. Amino acids. J. biol. Chem. **210**, 481—489 (1954).
DIETRICH, L. S.: A rapid method for the determination of dihydroxyphenylalanine decarboxylase in animal tissues. J. biol. Chem. **204**, 587—591 (1953).
DILLARD, G. H. P., G. BRECHER, and E. P. CRONKITE: Separation, concentration, and transfusion of platelets. Proc. Soc. exp. Biol. (N.Y.) **78**, 796—799 (1951).
DUGGAN, D. E., and S. UDENFRIEND: The spectrophotofluorometric determination of tryptophan in plasma and of tryptophan and tyrosine in protein hydrolysates. J. biol. Chem. **223**, 313—319 (1956).
DUNN, M. S., H. F. SCHOTT, W. FRANKL, and L. B. ROCKLAND: Investigations of amino acids, peptides, and proteins: determination of apparent free tryptophan in blood by microbiological method. J. biol. Chem. **157**, 387—394 (1945).
EBLE, J. N., and R. M. BROOKER: A novel quantitative application of thin layer chromatography: Assay of tryptamine. Experientia (Basel) **18**, 524 (1962).
EFRON, M.: High voltage paper electrophoresis. In: I. SMITH (ed.), Chromatographic and electrophoretic techniques, p. 158—189. London 1960.
EGELHAAF, A.: Der Gehalt an freiem Tryptophan und Kynurenin bei den Genotypen a^+ und a von Ephestia kühniella während der Entwicklung. Z. Naturforsch. **12**b, 465—472 (1957).
ERSPAMER, V.: The enterochromaffin cell system and enteramin (5-hydroxytryptamin). R. C. sci. Farmitalia **1**, 1—193 (1954).
— Observations on the fate of indolalkylamines in the organism. J. Physiol. (Lond.) **127**, 118—133 (1955).
— Some observations on the fate of exogeneous 5-hydroxytryptamine (enteramine) in the rat. J. Physiol. (Lond.) **133**, 1—9 (1956).
—, and G. BERTACCINI: Observations on the antidiuretic action and the fate of 5-hydroxy-DL-tryptophan in the rat organism. Arch. int. Pharmacodyn. **137**, 6—23 (1962).
—, and G. BORETTI: Identification and characterization, by paper chromatography, of enteramine, octopamine, tyramine, histamine and allied substances in extracts of posterior salivary glands of octopoda and in other tissue extracts of vertebrates and invertebrates. Arch. int. Pharmacodyn. **88**, 296—332 (1951).
—, and M. B. NOBILI: Observations on the fate of 4-hydroxy-DL-tryptophan in the organism of the rat and man. Arch. int. Pharmacodyn. **137**, 24—38 (1962a).
— — Observations on the fate of DL-5-hydroxy-N-acetyltryptophan and DL-5-acetoxy-N-acetyltryptophan in the rat organism. Arch. int. Pharmacodyn. **139**, 433—446 (1962b).
FALCK, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. **56**, Suppl. 197 (1962).
FALES, H. M., and J. J. PISANO: Gas chromatography of biologically important amines. Analyt. Biochem. **3**, 337—342 (1962).
FEARON, W. R., and W. A. BOGGUST: Pigments derived from tryptophan: (1) Urorosein, (2) Tryptochrome. Biochem. J. **46**, 62—67 (1950).
FLODIN, P., and J. PORATH: Molecular sieve processes. In: E. HEFTMANN (ed.), Chromatography, p. 328—343. New York 1961.
FUCHS, A. R., and F. FUCHS: Urinary excretion of 5-hydroxyindoleacetic acid during the menstrual cycle. Dan. med. Bull. **9**, 39—45 (1960).
GAEDTKE, K., u. K. SCHREIER: Eine Methode zur Serotoninbestimmung im Urin. Clin. Chim. Acta **1**, 475—477 (1956).
GAYER, J.: Zum Nachweis biogener Amine. Biochem. Z. **328**, 39—43 (1956).
GELOTTE, B.: Gel filtration. Sorption properties of the bed material sephadex. J. Chromatog. **3**, 330—342 (1960).
GERNGROSS, O., K. VOSS u. H. HERFELD: Über eine hochempfindliche, streng tyrosinspezifische Farbreaktion auf para-substituierte Phenole. Ber. dtsch. chem. Ges. **66**, 435—442 (1933).
GLUCKMAN, M. I., and R. ABRAMS: A method for serotonin determination. Fed. Proc. **18**, 395 (1959).
GOBLE, A. J., D. R. HAY, and M. SANDLER: 5-Hydroxytryptamine metabolism in acquired heart-disease associated with argentaffin carcinoma. Lancet **1955 II**, 1016—1017.
GRAHAM, C. E., E. P. SMITH, S. W. HIER, and D. KLEIN: An improved method for the determination of tryptophane with p-dimethylaminobenzaldehyde. J. biol. Chem. **168**, 711—716 (1947).

HAIS, I. M., u. K. MACEK: Handbuch der Papierchromatographie, Bd. 1. Jena 1958.
HANSON, A.: Unpublished results 1964.
—, and F. SERIN: Determination of 5-hydroxyindoleacetic acid in urine and its excretion in patients with malignant carcinoids. Lancet **1955 II**, 1359—1361.
HARLEY-MASON, J., and A. A. P. G. ARCHER: Use of p-dimethylaminocinnamaldehyde as a spray reagent for indole derivatives on paper chromatograms. Biochem. J. **69**, 60 P (1958).
HECKER, E.: Verteilungsverfahren im Laboratorium. Weinheim 1955.
HEINRICH, P., u. W. SCHULER: Stabilisierte Diazoniumsalze als Reagenzien zur Bestimmung von Dioxybenzolderivaten. II. Über eine kolorimetrische Bestimmungsmethode des 1,2-Dioxybenzols, des Methylaminomethyl-[3,4-dioxy-phenyl]-carbinols und der α-Amino-β-[3,4-dioxyphenyl]-propionsäure. Helv. chim. Acta **31**, 320—330 (1948).
HESS, S. M.: Chemical estimation of tryptamine. In: J. H. QUASTEL (ed.), Methods in Medical Research, vol. 9, p. 175—177. Chicago 1961.
— B. G. REDFIELD, and S. UDENFRIEND: The effect of monoamine oxidase inhibitor and tryptophan on the tryptamine content of animal tissues and urine. J. Pharmacol. exp. Ther. **127**, 178—181 (1959).
— P. A. SHORE, and B. B. BRODIE: Persistence of reserpine action after the disappearance of drug from brain: effect of serotonin. J. Pharmacol. exp. Ther. **118**, 85—89 (1956).
—, and S. UDENFRIEND: A fluorometric procedure for the measurement of tryptamine in tissues . J. Pharmacol. exp. Ther. **127**, 175—177 (1959).
HOLMSTEDT, B., and G. LUNDGREN: Unpublished results 1964.
— V. J. A. VANDENHEUVEL, W. L. GARDINER, and E. C. HORNING: The separation and identification of tryptamine-related indole bases by gas chromatographic methods. Analyt. Biochem. **8**, 151—157 (1964).
HORNET, J. C., T. V. CASEY, and J. W. RUNYAN: False-positive urinary test for 5-hydroxyindoleacetic acid due to methocarbamol and mephenesin carbamate. New Engl. J. Med. **261**, 188—190 (1959).
JEPSON, J. B.: Paper chromatography of urinary indoles. Lancet **1955 II**, 1009—1011.
— Indoles and related Ehrlich reactors. In: I. SMITH (ed.), Chromatographic and electrophoretic techniques, vol. 1, p. 183—211. London 1960.
—, and B. J. STEVENS: A fluorescence test for serotonin and other tryptamines. Nature (Lond.) **172**, 772 (1953).
— P. ZALTZMAN, and S. UDENFRIEND: Microsomal hydroxylation of tryptamine, indoleacetic acid and related compounds, to 6-hydroxy derivatives. Biochim. biophys. Acta (Amst.) **62**, 91—102 (1962).
KAKIMOTO, Y., and M. D. ARMSTRONG: The phenolic amines of human urine. J. biol. Chem. **237**, 208—214 (1962).
KEULEMANS, A. I. M.: Gas chromatography, 2 ed. New York 1959.
KIRBERGER, E.: Differentialdiagnostische Überlegungen bei vermehrter 5-Hydroxyindolessigsäure-Ausscheidung im Harn. Dtsch. med. Wschr. **87**, 829—834 (1962).
—, u. L. BRAUN: Über das Vorkommen von 5-Hydroxytryptamin in der Walnuß. Biochim. biophys. Acta (Amst.) **49**, 391 (1961).
LANGEMANN, H., u. J. KÄGI: Oxytryptamin- und Oxyindolessigsäurebestimmungen bei einem Fall von Carcinoidsyndrom, nebst einigen anderen Untersuchungen über Oxytryptamin. Klin. Wschr. **34**, 237—241 (1956).
LEDERER, M.: A introduction to paper electrophoresis and related methods. Amsterdam 1955.
LERNER, A. B., J. D. CASE, K. BIEMANN, R. V. HEINZELMAN, J. J. SZMUSZKOVICZ, W. C. ANTHONY, and A. KRIVIS: Isolation of 5-methoxyindole-3-acetic acid from bovine pineal glands. J. Amer. chem. Soc. **81**, 5264 (1959a).
— — and R. V. HEINZELMAN: Structure of melatonin. J. Amer. chem. Soc. **81**, 6084 (1959b).
LINSKENS, H. F.: Papierchromatographie in der Botanik. Berlin-Göttingen-Heidelberg 1959.
LOVENBERG, W., H. WEISSBACH, and S. UDENFRIEND: Aromatic L-amino acid decarboxylase. J. biol. Chem. **237**, 89—94 (1962).
MACEK, K.: Techniques of liquid-liquid partition chromatography. In: E. HEFTMANN (ed.), Chromatography, p. 112—163. New York 1961.
MACFARLANE, P. S., C. E. DALGLIESH, R. W. DUTTON, B. LENNOX, L. M. NYHUS, and A. N. SMITH: Endocrine aspects of argentaffinoma, with special reference to the use of the urinary 5-hydroxyindoleacetic acid estimations in diagnosis. Scot. med. J. **1**, 148—155 (1956).
MCILWAIN, H., and R. RODNIGHT: Practical neurochemistry. London 1962.
MCISAAC, W. M., and I. H. PAGE: The metabolism of serotonin (5-hydroxytryptamine). J. biol. Chem. **234**, 858—864 (1959).
MEAD, J. A., and K. F. FINGER: A simple extraction method for the determination of both norepinephrine and serotonin in brain. Biochem. Pharmacol. **6**, 52—53 (1961).

MOHLER, D. N.: Evaluation of the urine test for serotonin metabolites. J. Amer. med. Ass. **163**, 1138 (1957).
MORRIS, C. J. O. R., and P. MORRIS: Separation Methods in Biochemistry. London 1963.
MÜLLER, R.: Zur quantitativen Bestimmung von Indolylessigsäure mittels Papierchromatographie und Papierelektrophorese. Beitr. Biol. Pflanzen **30**, 1—32 (1953).
NÖLLER, H. G., u. P. STELGENS: Die hochspannungselektrophoretische Abtrennung des Serotonins von den übrigen xanthydrolpositiven Substanzen. Clin. chim. Acta **2**, 304—306 (1957).
OATES, J. A.: Measurement of urinary tryptamine, tyramine, and serotonin. In: J. H. QUASTEL (ed.), Methods in medical research, vol. 9, p. 169—174. Chicago 1961.
PITKÄNEN, E., M. M. AIRAKSINEN, O. O. MUSTALA, and J. PALOHEIMO: Observations on the specificity of the urinary 5-hydroxyindoleacetic acid determination with 1-nitroso-2-naphtol. Scand. J. clin. Lab. Invest. **14**, 571—577 (1962).
PROCHÁZKA, Z., 1953: Cited after PROCHAZKA (1958).
— Indolderivate. In: I. M. HAIS u. K. MACEK (eds.), Handbuch der Papierchromatographie, vol. I, p. 585—592. Jena 1958.
QUAY, W. B.: Differential extractions for the spectrophotofluorometric measurement of diverse 5-hydroxy- and 5-methoxyindoles. Analyt. Biochem. **5**, 51—59 (1963).
RANDERATH, K.: Dünnschicht-Chromatographie. Weinheim 1962.
RAPPORT, M. M., A. A. GREEN, and I. H. PAGE: Serum vasoconstrictor (serotonin). IV. Isolation and characterization. J. biol. Chem. **176**, 1243—1251 (1948).
RIBEIRO, L. P., E. MITIDIERI, and O. R. AFFONSO: Paper Electrophoresis. Amsterdam 1961.
ROBERTSON, J. I. S., and T. M. ANDREWS: Free serotonin in human plasma. Qualitative and quantitative estimation. Lancet **1961 I**, 578—580.
RODNIGHT, R.: Separation and characterization of urinary indoles resembling 5-hydroxytryptamine and tryptamine. Biochem. J. **64**, 621—626 (1956).
ROSS, G., I. B. WEINSTEIN, and B. KABAKOW: The influence of phenothiazine and some of its derivatives on the determination of 5-hydroxyindoleacetic acid in urine. Clin. Chem. **4**, 65—76 (1958).
SANDLER, M.: The determination of hydroxyindoles in biological materials. In: H. VARLEY and A. H. GOWENLOCK (eds.), The clinical chemistry of monoamines, p. 116—130. Amsterdam 1963.
— P. J. SCHEUER, and P. J. WATT: 5-Hydroxytryptophan-secreting bronchial carcinoid tumour. Lancet **1961 II**, 1067—1069.
—, and P. J. D. SNOW: An atypical carcinoid tumour secreting 5-hydroxy-tryptophan. Lancet **1958 I**, 137—139.
SCHANBERG, S. M.: A study of the transport of 5-hydroxytryptophan and 5-hydroxytryptamin (serotonin) into brain. J. Pharmacol. exp. Ther. **139**, 191—200 (1963).
SCHLOSSBERGER, H. G., u. H. KUCH: Synthese von 5,6-Dihydroxytryptophan und verwandten Verbindungen. Justus Liebigs Ann. Chem. **662**, 132—138 (1963).
— — u. I. BUHROW: Abtrennung von Indol-Derivaten aus Urin mittels Sephadex G 25. Hoppe-Seylers Z. physiol. Chem. **333**, 152—153 (1963).
SCHÖN, H., W. ORDNUNG u. E. SCHMID: Zum Nachweis der 5-Hydroxyindolessigsäure im Harn. Klin. Wschr. **38**, 405—406 (1960).
SCHREIER, K., u. K. GAEDTKE: Über die papierchromatographische Bestimmung von Serotonin und anderen Indolkörpern. Naturwissenschaften **43**, 130 (1956).
SCHWEIGERT, B. S., H. E. SAUBERLICH, C. A. ELVEHJEM, and C. A. BAUMANN: Free tryptophan in blood and urine. J. biol. Chem. **164**, 213—221 (1946).
SCOTT, T. A.: An enzymic method for the estimation of L-tryptophan. Biochem. J. **80**, 462—464 (1961).
SHEPHERD, D. M., G. B. WEST, and V. ERSPAMER: Detection of 5-hydroxytryptamine by paper chromatography. Nature (Lond.) **172**, 357 (1953).
SHORE, P. A., and J. S. OLIN: Identification and chemical assay of norepinephrine in brain and other tissues. J. Pharmacol. exp. Ther. **122**, 295—300 (1958).
SJOERDSMA, A.: Clinical and laboratory features of malignant carcinoid. Arch. intern. Med. **102**, 938—940 (1958).
— J. A. OATES, P. ZALTZMAN, and S. UDENFRIEND: Identification and assay of urinary tryptamine; application as an index of monoamine oxidase inhibition in man. J. Pharmacol. exp. Ther. **126**, 217—222 (1959).
— — — — Serotonin synthesis in carcinoid patients. Its inhibition by α-methyl-DOPA, with measurement of associated increases in urinary 5-hydroxytryptophan. New Engl. J. Med. **263**, 585—588 (1960).
— L. L. TERRY, and S. UDENFRIEND: Malignant carcinoid, a new metabolic disorder. Arch. intern. med. **99**, 1009—1012 (1957).
— H. WEISSBACH, and S. UDENFRIEND: Simple test for diagnosis of α-metastatic carcinoid (argentaffinoma). J. Amer. med. Ass. **159**. 397 (1955).

SMITH, I. (ed.): Chromatographic and electrophoretic techniques. London 1960.
SPRINCE, H., G. R. ROWLEY, and D. JAMESON: Spectrofluorometric studies of 5-hydroxyindoles and related compounds. Science **125**, 442—443 (1957).
STAHL, E. (ed.): Dünnschicht-Chromatographie. Berlin-Göttingen-Heidelberg: Springer 1962.
—, u. H. KALDEWEY: Spurenanalyse physiologisch aktiver einfacher Indolderivate. Hoppe-Seylers Z. physiol. Chem. **323**, 182—191 (1961).
STRELL, M., u. A. KALOJANOFF: Polymethinfarbstoffe. V. Mitt. Eine Erweiterung der Ehrlichschen Reaktion. Chem. Ber. **87**, 1025—1032 (1954).
STURM, A.: Die Bestimmung des Serotonins in Serum mit Hilfe der Hochspannungselektrophorese. Klin. Wschr. **39**, 365—367 (1961).
— Die Bestimmung der 5-Hydroxyindolessigsäure mit Hilfe der Hochspannungselektrophorese. Clin. chim. Acta **7**, 714—721 (1962).
SWEELEY, C. C., and C. M. WILLIAMS: Microanalytical determination of urinary aromatic acids by gas chromatography. Analyt. Biochem. **2**, 83—86 (1961).
TITUS, E. O.: Countercurrent distribution. In: C. D. STEWART, and A. STOLMAN (eds.), Toxicology, vol. 1, p. 392—422. New York 1960.
—, and S. UDENFRIEND: Metabolism of 5-hydroxytryptamine (serotonin). Fed. Proc. **13**, 411 (1954).
TRUTER, E. V.: Thin film chromatography. London 1963.
UDENFRIEND, S.: Fluorescence assay in biology and medicine. New York 1962.
— D. F. BOGDANSKI, and H. WEISSBACH: Fluorescence characteristics of 5-hydroxytryptamine (serotonin). Science **122**, 972—973 (1955a).
— D. E. DUGGAN, B. M. VASTA, and B. B. BRODIE: A spectrophotofluorometric study of organic compounds of pharmacological interest. J. Pharmacol. exp. Ther. **120**, 26—32 (1957b).
— W. LOVENBERG, and A. SJOERDSMA: Physiologically active amines in common fruits and vegetables. Arch. Biochem. **85**, 487—490 (1959).
—, and R. E. PETERSON: Assay of aromatic amino acids. III. Tryptophan. In: S. P. COLOWICK and N. O. KAPLAN (eds.), Methods in enzymology, vol. 3, p. 613—614. New York 1957.
— E. TITUS, and H. WEISSBACH: The identification of 5-hydroxy-3-indoleacetic acid in normal urine and a method for its assay. J. biol. Chem. **216**, 499—505 (1955c).
—, and H. WEISSBACH: 5-Hydroxytryptophan and derivatives. In: S. P. COLOWICK, and N. O. KAPLAN (eds.), Method in enzymology, vol. 6, p. 598—605. New York 1963.
— — and D. F. BOGDANSKI: Increase in tissue serotonin following administration of its precursor 5-hydroxytryptophan. J. biol. Chem. **224**, 803—810 (1957a).
— — and B. B. BRODIE: Assay of serotonin and related metabolites, enzymes, and drugs. In: D. GLICK (ed.), Methods of Biochemical Analysis, vol. 6, p. 95—130. New York 1958.
— — and C. T. CLARK: The estimation of 5-hydroxytryptamine (serotonin) in biological tissues. J. biol. Chem. **215**, 337—344 (1955b).
WAALKES, T. P.: The determination of serotonin (5-hydroxytryptamine) in human blood. J. Lab. clin. Med. **53**, 824—829 (1959).
— A. SJOERDSMA, C. R. CREVELING, H. WEISSBACH, and S. UDENFRIEND: Serotonin, norepinephrine and related compounds in banana. Science **127**, 648—650 (1958).
WEISSBACH, H., W. KING, A. SJOERDSMA, and S. UDENFRIEND: Formation of indole-3-acetic acid and tryptamine in animals. A method for estimation of indole-3-acetic acid in tissues. J. biol. Chem. **234**, 81—86 (1959).
— T. P. WAALKES, and S. UDENFRIEND: A simplified method for measuring serotonin in tissues; simultaneous assay of serotonin and histamine. J. biol. Chem. **230**, 865—871 (1958).
WEST, G. B.: Tryptamines in edible fruits. J. Pharm. Pharmacol. **10**, 589 (1958).
— Carcinoid tumours and pineapples. J. Pharm. Pharmacol. **12**, 768 (1960).
WIEGAND, R. G., and E. SCHERFLING: Determination of 5-hydroxytryptophan and serotonin. J. Neurochem. **9**, 113—118 (1962).
WOLLISH, E. G., M. SCHMALL, and M. HAMRYLYSHYN: Thin-layer chromatography. Recent development in equipment and applications. Analyt. Chem. **33**, 1138—1142 (1961).
ZYLKA, W., u. T. KÖHLER: Anwendung verschiedener Methoden zum quantitativen Nachweis von 5-Hydroxytryptamin und 5-Hydroxyindolessigsäure bei einem Fall von metastasierendem Dünndarmcarcinoid. Klin. Wschr. **35**, 622—626 (1957).