Zn^{2+} and Cu^{2+} ions. No absolute requirement for bivalent ions could be demonstrated with *Zwischenferment* from *A. niger*, and ethylenediaminetetraacetate (7×10^{-3} M) was found to have no effect on activity. The metal content of the enzyme preparation was, however, not investigated.

Cysteine, iodoacetate and fluoride had no effect on enzyme activity at a concentration of 10^{-2} M. The enzyme from liver has been reported to be susceptible to sulphydryl inhibitors (Glock & McLean, 1953).

DISCUSSION

Maximum *Zwischenferment* activity was obtained at the concentrations of G 6-P and TPN used in the routine assay system. Under the experimental conditions described by Kornberg (1950) at 25°, the activity of the enzyme was about 28 % less.

The purity of fraction IV was about six times higher than that reported by Kornberg for the yeast enzyme. The purified preparation of Negelein & Gerischer (1936) catalysed the uptake of 15·6 μmoles of oxygen/min./mg. of enzyme at 38°, when the activity was measured manometrically in the presence of Warburg's 'yellow enzyme' and oxygen, 1 μmole of oxygen being equivalent to 1 μmole of reduced TPN in the absence of catalase (Warburg & Christian, 1933). But the relative purity of this preparation is difficult to ascertain with accuracy owing to the differences in the method of enzyme assay.

SUMMARY

1. *Zwischenferment* from *Aspergillus niger* was purified about 60-fold.

2. The purified enzyme was free from phosphoglucoisomerase, phosphoglucomutase, hexokinase and 6-phosphogluconic dehydrogenase, and was suitable for analytical purposes.

3. Some of the properties of the enzyme, pH optimum, Michaelis constant, etc., have been described.

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Two-dimensional Paper Chromatography of Urinary Indoles and Related Substances

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Many indolic compounds, and their degradation products, can occur in urine in pathological states, e.g. carcinoidosis (cf. review by Langemann, 1955) and Hartnup syndrome (Dent, 1952). Some of the metabolites may be concerned with bladder cancer (e.g. Boyland & Williams, 1955), and the appearance of metabolites in the urine may give valuable information on the state of vitamin nutrition (Dalgliesh, 1956). Identification of such substances is thus becoming of increasing importance, but the number of indolic compounds and their degradation products, potentially occurring, is too large to permit separation on one-dimensional chromatograms. A two-dimensional system suitable for routine use was therefore sought, and this paper reports the 'map of the spots' given by the solvent combination finally selected from amongst several tried.

EXPERIMENTAL

Chromatograms were run by the descending technique on sheets of Whatman no. 1 paper, $18\frac{1}{2}$ in. $\times 22\frac{1}{2}$ in. The sources of substances used in synthetic mixtures are given in the footnotes to Tables 1 and 2. Urine extracts were prepared by adsorption of aromatic metabolites on deactivated charcoal, followed by elution with aqueous phenol, and concentration of the phenol eluate (Dalgliesh, 1955*a*). All chromatograms were run at room temperature, the chromatography tanks being away from draughts, but the temperature not being otherwise controlled.

Chromatograms were first run in the long dimension overnight (about 15 hr.), the organic layer of a freshly prepared *n*-butanol–acetic acid–water mixture (4:1:5, by vol.; Partridge, 1946) being used as solvent. The chromatograms were dried and then run in the short dimension with 20% (w/v) aqueous KCl as solvent (Boscott, 1952). The run in the second dimension requires only 3–4 hr.

The detection and colour reactions of most of the substances in Tables 1 and 2 have been described in earlier papers (Dalgliesh, 1952*b*, 1955*a*, *c*). Colour reactions of the remaining substances can be readily inferred by comparing their structures with those of similar substances described in the above-mentioned papers.

RESULTS AND DISCUSSION

Previous work (Dalgliesh, 1952*b*) showed that for a single-dimensional separation of indoles and related substances an excellent solvent is butanol–acetic acid–water. This mixture was therefore selected for separation in the first dimension and search was made for the most suitable solvent for the second dimension. A basic solvent is undesirable because of the ready oxidation of many relevant substances (e.g. 5-hydroxyindolylacetic acid, 3-hydroxykynurenine) under alkaline conditions. Propan-2-ol–ammonia has been used by Jepson (1955) for chromatography of urinary indoles, but in our experience there was appreciable oxidation with this solvent, and moreover the derived two-dimensional pattern obtained with known substances and with urine extracts was not well spaced out. Other basic solvents tried had similar defects. Homogeneous alcohol solutions (e.g. propan-2-ol–water) gave poor resolution, and the volatility of lower alcohols resulted in variable R_F values when chromatography was carried out at room temperature without temperature control. Phenolic solvents are slow, and undesirable when reagents for phenols are likely to be used for detecting metabolites. Neither aqueous alcohols nor aqueous phenols gave good two-dimensional patterns when combined with butanol–acetic acid–water as first solvent. However, excellent results were obtained with aqueous salt solutions (Boscott, 1952). These give good resolution, the chromatograms need no pre-equilibration and, when chromatograms are carried out at room temperature without temperature control, aqueous salt solutions result in more consistent R_F values than are usually given by mixtures containing organic solvents. Moreover, salt solutions are fast-running, and enable a two-dimensional chromatogram on standard-sized sheets (18½ in. × 22½ in.) to be completed in 18–20 hr. Curzon (1955) has also found an aqueous salt solution to be a good chromatographic solvent for indoles.

Several salt solutions were tried. Salts of organic acids, such as sodium formate, were found undesirable because the mildly alkaline conditions favoured oxidation. Neutral salts, such as sodium or potassium chloride or ammonium sulphate, all gave comparable chromatograms, the resultant R_F values tending to decrease as salt concentration increased. A 20% (w/v) aqueous solution of potassium chloride was finally selected for routine use and a standard 'map of the spots' prepared.

The results are summarized in Table 1. All R_F values in Table 1 are based on a minimum of ten determinations. R_F values of authentic substances were determined on mixtures of at least six, and usually ten to twelve, substances run simultaneously, and each substance was run in at least four different combinations. The chromatograms reported in Tables 1 and 2 were carried out during a period of about 6 months, without special control of temperature. The ranges quoted in Table 1 show the order of variability of R_F values encountered. The comparative constancy of R_F values obtained with the aqueous solvent is evident. The R_F values for substances in urine extracts were obtained at different times (and usually with different batches of paper) from those for the same substances in synthetic mixtures, and comparison of the two sets of values shows the order of constancy of the chromatographic behaviour. The resultant chromatographic pattern of the principal substances related to indolic metabolism is combined in the 'map of the spots' shown in Fig. 1. Some substances, notably skatole and *o*-aminoacetophenone, volatilize during the double drying involved in two-dimensional chromatography and are omitted from Table 1 and Fig. 1. Basic substances in the form of salts run more slowly in butanol–acetic acid, and values for both free bases and hydrochlorides are therefore given. Overlapping occurs between indolylaceturic and indolyl-lactic acids, and between indolylpropionic acid and indolylacetonitrile. There is no evidence as yet to suggest that indolylacetonitrile occurs other than in plant sources. Other pairs of substances running close together, e.g. pairs 3 and 7 or 5 and 22, differ sufficiently in their reactions to be readily distinguishable even if overlapping occurs.

Some of the values in Table 1 are for substances available only as components of urine extracts: most of these can be identified with confidence. G. Curzon (private communication) has found that each of several 5-hydroxyindoles investigated gives on autoxidation at pH 7 appreciable amounts of a new indole, the autoxidation product of 5-hydroxyindolylacetic acid being apparently identical with a major indolic constituent of the freshly passed urines of patients with carcinoidosis. These observations have been confirmed and the R_F values of the 5-hydroxyindolylacetic acid autoxidation product are included in Table 1.

Table 1. *Chromatographic behaviour of known or potential tryptophan metabolites and related indolic compounds*

Substance	Origin of mixture†	Ref. no. on map	No. of determinations	Mean R_F, butanol–acetic acid (range in parentheses)	Mean R_F, 20% KCl (range in parentheses)	Source of material
N^{α}-Acetylkynurenine	Synth.	1	10	0·83 (0·80–0·86)	0·82 (0·80–0·83)	A
	Urine	—	16	0·82 (0·76–0·86)	0·82 (0·81–0·83)	B
N^{α}-Acetyl-3-hydroxykynurenine	Urine	2	16	0·75 (0·70–0·81)	0·74 (0·70–0·76)	B
N^{α}-Acetyltryptophan	Synth.	3	11	0·86 (0·84–0·89)	0·68 (0·65–0·71)	C
o-Aminohippuric acid	Synth.	4	10	0·77 (0·72–0·82)	0·81 (0·79–0·82)	D
	Urine	—	12	0·76 (0·72–0·81)	0·80 (0·78–0·83)	E
2-Amino-3-hydroxyacetophenone	Synth.	5	10	0·87 (0·84–0·90)	0·51 (0·47–0·56)	F
2-Amino-3-hydroxyacetophenone *O*-sulphate	Synth.	6	10	0·46 (0·42–0·53)	0·71 (0·69–0·73)	G
	Urine	—	18	0·47 (0·43–0·52)	0·72 (0·70–0·76)	H
Anthranilic acid	Synth.	7	10	0·89 (0·87–0·93)	0·67 (0·64–0·68)	I
	Urine	—	12	0·88 (0·84–0·92)	0·67 (0·66–0·68)	E
Anthranilic acid glucuronide	Urine	8	12	0·57 (0·54–0·60)	0·81 (0·79–0·84)	E
Bufotenin (free base)	Synth.	9	10	0·50 (0·47–0·56)	0·51 (0·49–0·53)	J
Bufotenin (hydrochloride)	Synth.	9a	13	0·34 (0·32–0·38)	0·51 (0·48–0·57)	J
3-Hydroxyanthranilic acid	Synth.	10	12	0·85 (0·81–0·88)	0·61 (0·56–0·63)	C
5-Hydroxyanthranilic acid	Synth.	11	13	0·58 (0·53–0·61)	0·71 (0·69–0·75)	K
5-Hydroxyindolylacetic acid	Synth.	12	23	0·76 (0·73–0·79)	0·49 (0·46–0·51)	J
	Urine	—	44	0·74 (0·71–0·79)	0·48 (0·43–0·52)	H
5-Hydroxyindolylacetic acid autoxidation product	Synth.	13	10	0·50 (0·46–0·53)	0·85 (0·83–0·86)	Cf. text
	Urine	—	13	0·50 (0·45–0·55)	0·84 (0·83–0·85)	H
5-Hydroxyindolylacetic acid *O*-sulphate (presumed)	Urine	14	10	0·37 (0·33–0·45)	0·72 (0·68–0·76)	L
3-Hydroxy-L-kynurenine*	Synth.	15	11	0·31 (0·25–0·32)	0·65 (0·60–0·69)	C
	Urine	—	28	0·29 (0·28–0·37)	0·65 (0·62–0·71)	B
3-Hydroxykynurenine *O*-sulphate	Urine	16	16	0·11 (0·08–0·16)	0·80 (0·78–0·82)	B
5-Hydroxy-L-kynurenine*	Synth.	17	12	0·17 (0·15–0·22)	0·73 (0·67–0·77)	M
5-Hydroxytryptamine (free base)	Synth.	18	13	0·42 (0·37–0·45)	0·39 (0·36–0·41)	J, N
	Urine	—	10	0·42 (0·39–0·44)	0·37 (0·35–0·39)	H
5-Hydroxytryptamine (hydrochloride)	Synth.	18a	11	0·27 (0·23–0·30)	0·39 (0·36–0·41)	J, N
5-Hydroxytryptophan	Synth.	19	12	0·17 (0·15–0·20)	0·44 (0·42–0·47)	O, P
	Urine	—	10	0·15 (0·12–0·18)	0·43 (0·41–0·45)	H
Indican (indoxyl *O*-sulphate)	Synth.	20	20	0·40 (0·35–0·45)	0·65 (0·62–0·69)	Q
	Urine	—	53	0·43 (0·37–0·51)	0·64 (0·61–0·68)	H, R
Indole	Synth.	21	12	0·95 (0·92–0·97)	0·03 (0·00–0·06)	I
Indolylacetic acid	Synth.	22	36	0·88 (0·85–0·91)	0·55 (0·50–0·61)	I
	Urine	—	42	0·88 (0·84–0·93)	0·55 (0·50–0·60)	H, R, S
Indolylacetic acid glucuronide (presumed)	Urine	23	10	0·52 (0·47–0·60)	0·71 (0·69–0·72)	S
Indolylacetonitrile	Synth.	24	10	0·91 (0·88–0·94)	0·37 (0·32–0·41)	U
Indolylaceturic acid	Synth.	25	10	0·81 (0·77–0·85)	0·64 (0·60–0·69)	T
	Urine	—	25	0·81 (0·75–0·87)	0·63 (0·59–0·70)	S
Indolylacetylglutamine	Synth.	33	12	0·63 (0·59–0·67)	0·70 (0·67–0·73)	X
Indolyl-lactic acid	Synth.	26	18	0·77 (0·73–0·82)	0·62 (0·58–0·66)	W
Indolylpropionic acid	Synth.	27	12	0·92 (0·90–0·93)	0·40 (0·37–0·43)	I
L-Kynurenine*	Synth.	28	11	0·37 (0·32–0·42)	0·73 (0·72–0·78)	A
	Urine	—	28	0·37 (0·33–0·43)	0·75 (0·71–0·77)	B
Kynurenic acid	Synth.	29	10	0·54 (0·50–0·58)	0·41 (0·38–0·44)	I
	Urine	—	16	0·58 (0·53–0·62)	0·41 (0·38–0·43)	B
Tryptamine (free base)	Synth.	30	13	0·64 (0·59–0·67)	0·53 (0·51–0·56)	I
Tryptamine (hydrochloride)	Synth.	30a	11	0·50 (0·45–0·53)	0·53 (0·51–0·57)	I
Tryptophan	Synth.	31	23	0·41 (0·35–0·45)	0·59 (0·55–0·63)	I
	Urine	—	40	0·41 (0·32–0·48)	0·59 (0·54–0·62)	B, H, R
Xanthurenic acid	Synth.	32	13	0·54 (0·51–0·57)	0·28 (0·24–0·31)	V
	Urine	—	16	0·58 (0·55–0·60)	0·24 (0·22–0·25)	B

* The D- and L-forms separate on paper chromatography (Dalgliesh, 1952*c*). † Synth: a synthetic mixture of known substances.
Sources: A, synthetic (Dalgliesh, 1952*a*); B, from urine of tryptophan-fed pyridoxine-deficient rats (Dalgliesh, 1952*b*); C, synthesized by standard procedures; D, synthetic (Charconnet-Harding, Dalgliesh & Neuberger, 1953); E, from urine of anthranilic acid-fed normal rats (Charconnet-Harding *et al.* 1953); F, gift of Professor Musajo (Musajo, Spada & Casini, (1950); G, synthetic (Dalgliesh, 1955*b*); H, from pathological human urines; I, commercial; J, gift of Drs M. E. Speeter and D. A. Shepherd, Upjohn Co.; K, gift of Dr W. V. Thorpe; L, presumed identification (Snow, Lennard-Jones, Curzon & Stacey, 1955) of substance in carcinoid urine; M, gift of Professor A. Butenandt; N, gift of Dr H. Holgate, Sandoz Products Ltd.; O, gift of Dr A. Cohen, Roche Products Ltd.; P, gift of Dr G. J. Martin, National Drug Co.; Q, gift of Dr P. Sims (Boyland, Sims & Williams, 1956); R, from normal human and animal urines; S, from urine of indolylacetic acid-fed normal rats; T, gift of Professor T. Wieland (Wieland & Hörlein, 1955); U, gift of Professor E. R. H. Jones (Henbest, Jones & Smith, 1953); V, gift of Dr W. E. Knox; W, gift of Dr M. D. Armstrong (Armstrong & Robinson, 1954); X, gift of Dr J. B. Jepson (Jepson, 1956).

Table 2. *Chromatographic behaviour of some miscellaneous substances, mostly aromatic*

Substance	Origin of mixture*	No. of determinations	Mean R_F, butanol–acetic acid (range in parentheses)	Mean R_F, 20% KCl (range in parentheses)	Source of material
2-Carboxypyrrole	Synth.	10	0·86 (0·83–0·89)	0·67 (0·63–0·72)	A
2:5-Dihydroxyphenylacetic (homogentisic) acid	Synth.	10	0·76 (0·72–0·81)	0·76 (0·75–0·78)	B
3:4-Dihydroxyphenylalanine	Synth.	10	0·17 (0·16–0·23)	0·76 (0·74–0·77)	A
m-Hydroxybenzoic acid	Synth.	10	0·89 (0·87–0·92)	0·60 (0·58–0·65)	A
o-Hydroxyphenylalanine (*o*-tyrosine)	Synth.	12	0·40 (0·37–0·43)	0·82 (0·80–0·83)	B
Phenylalanine	Synth.	12	0·50 (0·47–0·53)	0·88 (0·86–0·92)	A
Phenylethylamine (free base)	Synth.	13	0·72 (0·68–0·75)	0·82 (0·79–0·85)	A
Phenylethylamine (hydrochloride)	Synth.	11	0·66 (0·60–0·71)	0·83 (0·79–0·87)	A
Porphobilinogen	Synth.	11	0·42 (0·39–0·50)	0·86 (0·85–0·88)	C
Riboflavin	Synth.	25	0·22 (0·17–0·27)	0·43 (0·39–0·48)	A
	Urine	10	0·23 (0·19–0·25)	0·45 (0·43–0·47)	D
Riboflavin 5-phosphate	Synth.	12	0·06 (0·03–0·10)	0·55 (0·50–0·60)	A
Salicylic acid	Synth.	10	0·91 (0·90–0·92)	0·60 (0·54–0·63)	A
Salicyluric acid	Synth.	10	0·85 (0·82–0·87)	0·68 (0·65–0·70)	E
Tyramine (free base)	Synth.	11	0·57 (0·52–0·62)	0·80 (0·78–0·83)	A
Tyramine (hydrochloride)	Synth.	11	0·44 (0·37–0·49)	0·79 (0·77–0·82)	A
Tyrosine	Synth.	10	0·32 (0·28–0·41)	0·82 (0·80–0·83)	A
Tyrosine *O*-sulphate	Synth.	12	0·12 (0·08–0·17)	0·89 (0·87–0·92)	F
Urea	Synth.	12	0·49 (0·44–0·55)	0·86 (0·84–0·88)	A
	Urine	10	0·49 (0·45–0·56)	0·86 (0·84–0·87)	D

* Synth: a synthetic mixture of known substances.

Sources: A, commercial; B, gift of Professor A. Neuberger; C, gift of Professor C. Rimington; D, from human or animal urines; E, synthesized by Dr P. Wright (Bondi, 1907); F, gift of Dr F. R. Bettelheim (Bettelheim, 1954).

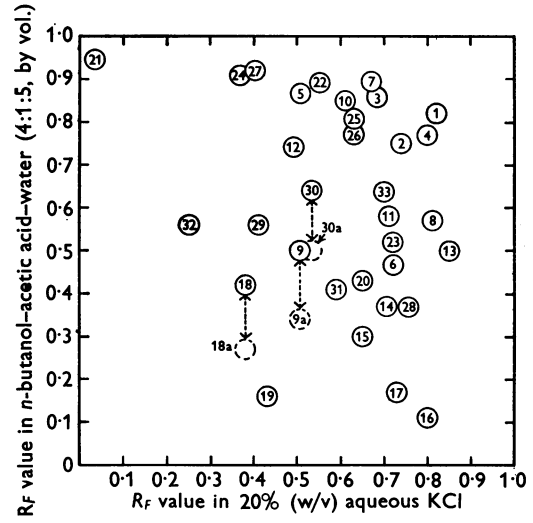

Fig. 1. 'Map of the spots' showing the pattern given by known tryptophan metabolites and related substances in the chromatographic system described in the text. Numbers refer to Table 1. The broken lines indicate the retardation of the bases when they are run as hydrochlorides.

Comparison of related substances in Table 1 suggests the existence of relationships between structure and R_F values which might be helpful in identifying unknown substances. Thus, in the cases examined, sulphate conjugation of a phenol (cf. pairs 5 and 6; 12 and 14; 15 and 16) or glycine conjugation of an acid (cf. pairs 4 and 7; 22 and 25) resulted in a decrease in R_F value in butanol–acetic acid with an increase in R_F value in the aqueous solvent, whereas introduction of a phenolic group (cf. pairs 1 and 2, 12 and 22, 18 and 30) caused a decrease in R_F in butanol–acetic acid and usually also in KCl, the degree of retardation varying with the position of the phenolic group (cf. trios 7, 10 and 11; 15, 17 and 28).

The chromatographic properties of some aromatic substances not connected with the metabolism of indoles are summarized in Table 2. Some of these, e.g. riboflavin and salicylic acid, are useful as fluorescent markers. The present solvent combination is not suitable for substances related to phenylalanine and tyrosine metabolism, as these all tend to run fast in the aqueous solvent. The rate of movement in salt solution appears to depend more on the aromatic nucleus than on the side chain, e.g. indolylacetic acid, tryptophan and tryptamine all have similar R_F values in this dimension, lower than the R_F values of tyramine and tyrosine, which in turn are lower than the R_F values of phenylalanine and phenylethylamine. The tendency to separate benzenoid from indolic compounds is of course an advantage in studying tryptophan metabolites.

Figs. 2 and 3 show typical chromatograms of urine extracts separated in the system described above. Fig. 2 shows the substances reacting with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in aqueous HCl) in the phenol eluate of a deactivated charcoal

adsorbate of urine (Dalgliesh, 1955*a*) from a case of carcinoidosis. The particular case illustrated (to be described elsewhere) is unusual in that both 5-hydroxytryptamine and 5-hydroxytryptophan are excreted in considerable amounts, as well as 5-hydroxyindolylacetic acid. However, most or all of the other substances shown in Fig. 2 have been observed in each of several carcinoid urines examined. Fig. 3 shows the tryptophan metabolites in a similarly prepared extract of the urine of rats deficient in pyridoxine and fed on tryptophan. Two-dimensional chromatography reveals small amounts of several substances not observed on one-dimensional chromatography (Dalgliesh, 1952*b*).

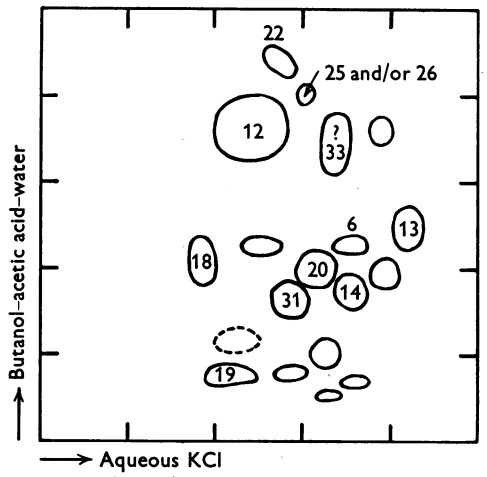

Fig. 2. Distribution of indolic and related compounds (full lines) excreted in a case of carcinoidosis, as revealed by the chromatographic system described. Numbers of identified spots refer to Table 1. Remainder are unidentified substances reacting with Ehrlich's reagent. The broken line indicates the position of riboflavin, present in the urine and useful as a marker.

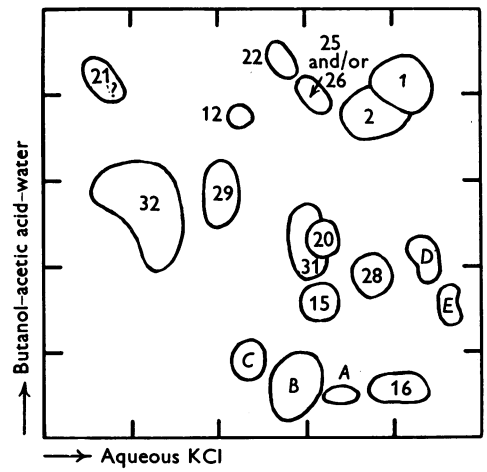

Fig. 3. Distribution of metabolites from the urine of tryptophan-fed pyridoxine-deficient rats. Numbers of identified spots refer to Table 1. Identity of remainder not established, but *A* is probably indolic, *B* and *C* are probably conjugates of xanthurenic or kynurenic acid or both, and *D* and *E* are probably conjugates or derivatives of 2-amino- or 2-amino-3-hydroxy-acetophenone.

SUMMARY

1. Indoles and other metabolites of tryptophan can be conveniently separated on two-dimensional paper chromatograms with the organic layer of a butanol–acetic acid–water mixture (4:1:5, by volume) as first solvent and 20% (w/v) aqueous potassium chloride as second solvent.

2. The chromatographic behaviour of nearly fifty compounds is described, and the chromatographic pattern of the principal substances related to tryptophan metabolism is summarized in a 'map of the spots'.

I thank the many individuals and firms (indicated in the footnotes to Tables 1 and 2) who have given me authentic substances; and Mr A. Asatoor for skilled assistance.

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