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AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

III. DETECTION AND IDENTIFICATION BY SELECTIVE STAINING

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SUMMARY

A simple and rapid method is described for the detection and unambiguous identification of seventy-six nitrogen-containing metabolites which are commonly found in biological fluids. Following the separation of these compounds by thin-layer chromatography they are identified by means of selective staining reagents.

INTRODUCTION

In earlier papers[1,2] (Parts I and II) the separation and quantitative determination of the common amino acids have been described. Nevertheless because of the large number of amino acids and related nitrogen-containing metabolites which occur in biological fluids, their unambiguous identification after separation by thin-layer chromatography (TLC), continues to present many difficulties within the concentration range of 5×10^{-4} to 2×10^{-2} μmole. The simplest approach[3,4] aims at ensuring that each amino acid occupies a unique position on the thin-layer chromatogram. This approach fails in the case of complex biological materials which may contain over seventy nitrogen-containing compounds as well as sugars and inorganic salts. Another approach favours the use of multiple solvent systems or repeated development[5–7]. It is our opinion that such methods are tedious and possess few attributes for general use.

A different approach to the problem involves the use of selective staining reagents in order to identify each amino acid unambiguously. This technique can be very effective in conjunction with solvent systems[1,2] which are capable of resolving many amino acids. The two-dimensional solvent system of HAWORTH AND HEATHCOTE[1] clearly resolved some forty amino acids and the positions of some twenty-three additional amino acids of less frequent occurrence were also noted. Ninhydrin is still the most widely used sensitive reagent for amino acids and there have been many variations of reagent composition, including the incorporation of metal salts into the

ninhydrin solution in order to enhance the stability of the resulting amino acid complex and to give some measure of specificity. Extensive lists of amino acid staining reagents have been given by STAHL[8], DAWSON *et al.*[9], and by KRAUSS[10].

The present paper describes the chromatographic behaviour of seventy-six compounds of biochemical interest and also lists selective staining reagents which permit their unambiguous identification. The compounds include the sixty-three substances listed by HAWORTH AND HEATHCOTE[1], and their sequence of numbering is retained.

EXPERIMENTAL

Materials and equipment

The TLC equipment, glass tanks, and 1 μl "microcaps" capillary pipettes were supplied by Shandon*.

Preparation of the cellulose layers. The purified cellulose powder MN300** (15 g) was spread over five plates (20 × 20 cm) at a thickness of 400 μ. After coating the plates with the cellulose slurry, the plates were allowed to dry horizontally overnight before use. The conditions for the purification of the powder, spreading the slurry, application of the sample solutions (1 μl) and development were those described previously[1].

Standard solutions. Stock solutions (0.025 *M*) of amino acids and other common nitrogen-containing metabolites were prepared using aqueous 2-propanol (10% v/v) as the solvent. In the case of tyrosine and other sparingly soluble amino acids, the minimum quantity of dilute hydrochloric acid was added to effect solution. These stock solutions and a range of solutions (0.025–0.0005 *M*) prepared by dilution were kept refrigerated when not in use.

Chromatographic solvent systems. The solvent systems which were used for the separation of the nitrogen-containing metabolites were those described by HAWORTH AND HEATHCOTE[1]. These were, for the first dimension (Solvent No. 1): 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25, v/v), and for the second dimension (Solvent No. 2): 2-methylpropanol-2–butanone–propanone–methanol–water (0.88) ammonia (40 : 20 : 20 : 1 : 14 : 5, v/v).

Application of samples and development of plates

In general the conditions of development which were followed were those previously described[1] but the first solvent front was not isolated when indoles of high R_F values were suspected to be present. Briefly the conditions were as follows:

Standard solutions (1 μl) were applied at a position 1.5 cm from both the bottom and left hand edges of the coated plate. The plates were then placed in chromatographic tanks containing 100 ml of Solvent No. 1, or 170 ml of Solvent No. 2. The atmosphere in each tank had been pre-saturated with the appropriate solvent before use, as described by SANKOFF AND SOURKES[11]. After the ascending solvent front due to Solvent No. 1 had travelled 13 cm from the origin, the plate was removed and dried in a stream of cold air for 15 min. Traces of acid impair the subsequent identification of the amino acids and, in order to remove all traces of hydrogen chloride, the air-dry

* Shandon Scientific Co., 65 Pound Lane, London, N.W. 10, Great Britain.
** Macherey, Nagel and Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge, Great Britain.

plate must be heated to 60° for a minimum of 15 min, or until no odour remains. The dry plate was then developed in Solvent No. 2 until the solvent front had reached a height of 13 cm from the origin, at which point the plate was removed and dried at 60° for 15 min.

Detection of amino acids and related compounds

The initial detection of the amino acids was effected by spraying the developed and dried plate with the cadmium acetate–ninhydrin reagent of HEATHCOTE AND WASHINGTON[12]. This reagent (designated Reagent A), was prepared from cadmium acetate (0.5 g), water (50 ml), glacial acetic acid (10 ml), and propanone to a final volume of 500 ml. Sufficient solid ninhydrin was added to suitable portions of this stable stock solution to give a ninhydrin concentration of 0.2% (w/v) for immediate

TABLE I

COMPOSITION AND CONDITIONS OF USE OF THE STAINING REAGENTS

Reagent	*Composition of the reagent*	*Reference*
A	Cadmium acetate–ninhydrin reagent.	12
B	Solution (1) was made from ethanol (50 ml), collidine (2 ml), glacial acetic acid (10 ml) and sufficient ninhydrin to give a 0.2% (w/v) solution. Solution (2) was a solution of copper nitrate (1% w/v) in ethanol. Mix solution (1) (50 ml) and solution (2) (3 ml). Spray the plate with the reagent and heat to 105° for 2 min.	13
C	This reagent was formulated as for Reagent A, except that isatin (0.2% w/v) replaced ninhydrin. After spraying, heat the plate at 90° for 10 min.	2
D_1	This was a solution of O-phthalaldehyde (0.2% w/v) in propanone.	14
D_2	A solution of potassium hydroxide (1% w/v) in ethanol. Spray with Reagent D_1 and heat at 50° for 20 min. Re-spray with Reagent D_2. Examine the plate at each stage of spraying under visible and UV light.	15
E	Solution of vanillin (2% w/v) in 1-propanol. Heat to 110° for 5 min and then re-spray with Reagent D_2. Examine the plate at each stage of spraying under both visible and UV light.	16
F	Reagent consists of *p*-dimethylaminobenzaldehyde (1 g) dissolved in conc. hydrochloric acid (5 ml) and then ethanol added to a final volume of 100 ml. Use at room temperature.	17
G	A solution of isatin (0.2% w/v) in a copper uranyl acetate solution. The latter was prepared from copper acetate (0.227 g), uranyl acetate (0.273 g), glacial acetic acid (20 ml), water (50 ml), and propanone to a total volume of 500 ml. After spraying, heat the plate to 90° for 10 min, examine under visible and UV light and then re-spray with Reagent F.	18
H	Aqueous solutions of sodium hydroxide (10% w/v), potassium ferricyanide (10% w/v) and sodium nitroprusside (10% w/v) are prepared. Equal volumes of each of the above solutions are mixed at 0°, allowed to stand at 0° for 30 min. Water (360 ml) is then added, and the reagent is stable at 0° for 1 month. Spray plate; colours appear within 5 min at room temperature.	19
I_1	Solution of pyridine (2% v/v) in methanol, to which add sufficient periodic acid to give a concentration of 0.03 *M*.	20
I_2	Ammonium acetate (15 g), glacial acetic acid (0.3 ml), acetylacetone (1 ml) and methanol to a total volume of 100 ml. After spraying the plate with Reagent I_1, leave at room temp. for 10 min; examine under visible and UV light. Re-spray plate with Reagent I_2 and examine as before.	
J	Solution (1): sodium nitroprusside (5% w/v) in methanol. Solution (2): piperidine (20% v/v) in methanol. Mix equal volumes of solutions (1) and (2) to form reagent which is unstable. Spray plate with Reagent I_1 and leave at room temp. for 5 min; examine under visible and UV light and then re-spray with Reagent J.	20

TABLE II

R_F VALUES OF ADDITIONAL[a] METABOLITES ON THIN LAYERS OF CELLULOSE

No.	*Compound*	*R_F (× 100)*	
		Solvent No. 1	*Solvent No. 2*
64	Saccharopine	73	0
65	Indican	89	91
66	5-Hydroxytryptophan	43	26
67	3-Indolyllactic acid	96	60
68	Tryptamine	73	97
69	3-Indolylacetic acid	98	56
70	Hippuric acid	71	0
71	Urea	70	50
72	Creatinine	47	41
73	Guanidine	50	36
74	Thiourea	71	70
75	Carnosine	9	21
76	Allantoin	49	9

[a] Additional to the 63 previously reported[1].

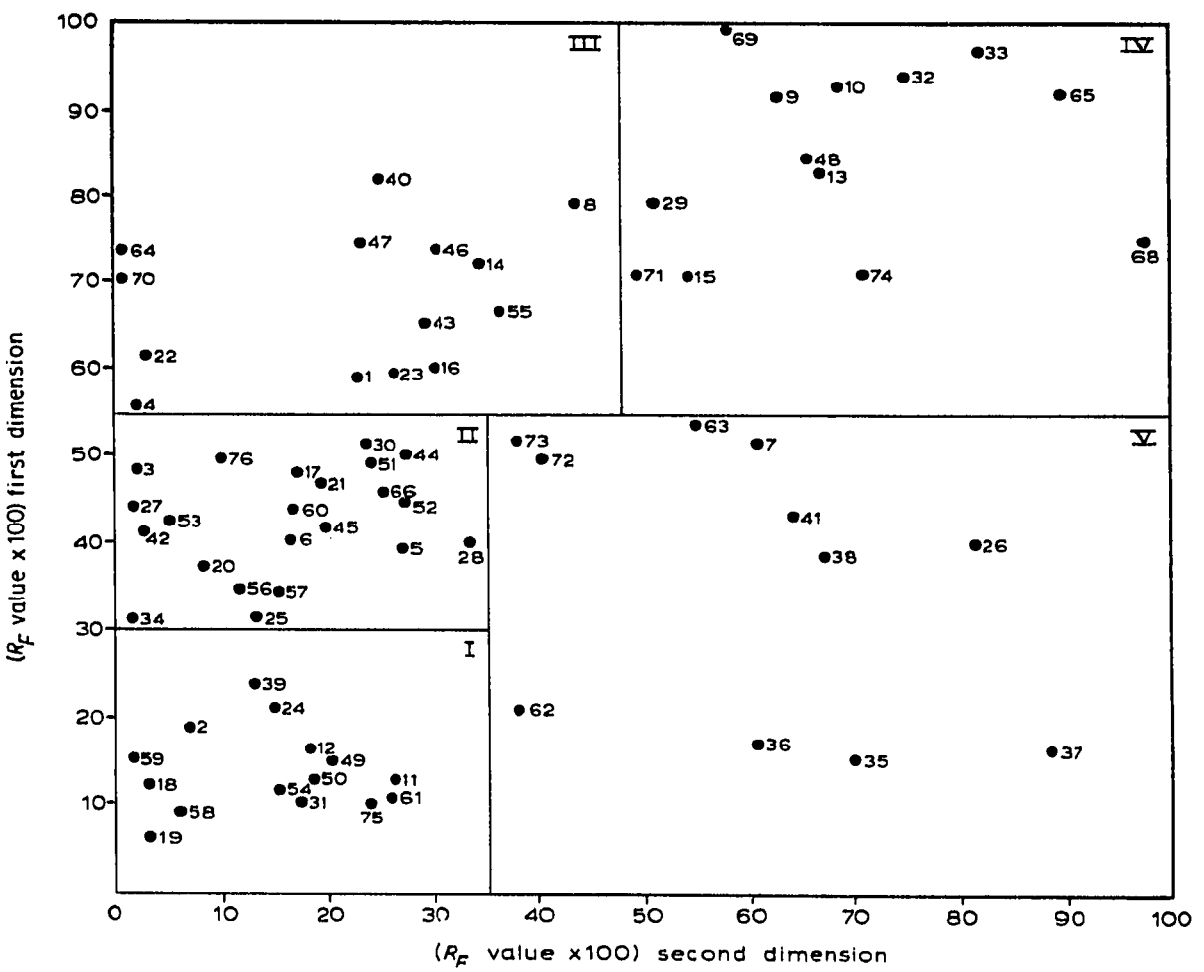

Fig. 1. Positions of metabolites on a TLC plate.

use. After the plates had been sprayed, they were heated to 60° for 15 min or left overnight at ambient temperature as convenient.

The final characterisation of the separated compounds was accomplished by the use of one or more staining reagents (Reagents B–J) listed in Table I. This table gives the composition of the reagents and the conditions under which they were used.

RESULTS AND DISCUSSION

The R_F values of thirteen compounds (Nos. 64–76) additional to the sixty-three reported by HAWORTH AND HEATHCOTE[1], are given in Table II. After separation by TLC, all the seventy-six compounds were arbitrarily arranged into five groups (I–V) on the one (20 × 20 cm) plate. See Fig. 1. The staining reactions of the individual metabolites are given for each group in Tables III–VII.

TABLE III

COLOUR REACTIONS OF METABOLITES OF GROUP I

The conditions which produce a positive response with Reagents D, E, G, I, J are indicated by superscripts: visible light and first spray reagent[1]; UV light and first spray reagent[2]; visible light and second spray reagent[3]; UV light and second spray reagent[4]. The colours produced are indicated by the following letter codes: Pink, P; Blue, Bl; Brown, Bn; Black, Bk; Yellow, Y; Orange, O; Red, R; Green, G; Grey, Gy; Purple, Pu; White, W; Yellow-Green (mixed colour), Y-G; faint, f; no reaction, —.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Arginine	2	R	Pu	R-Pu	Pu[1]	Y[3]	—	Pu[1]	P-O	—	—
Histidine	11	R	Pu	Bl-Gy	Pu[1] G[3]	Y[3]	—	Pu[2,3]	Pu	Y-G[4]	—
Lysine	12	R	Pu	Pu	Pu[1]	Y[3]	—	Pu[1]	—	—	—
Cysteine	18	Pu	Gy	Gy	Pu[1]	Y[3]	—	Pu[1]	—	—	—
Cystine	19	Pu	Bl	Pu	Pu[1]	Y[3]	—	Pu[1]	—	—	—
Asparagine	24	Bn	Bn	Pu	Pu[1]	Y[3]	—	Pu[1]	—	—	—
Hydroxylysine	31	R	Pu	Pu	Pu[1]	Y[3]	—	Pu[1]	—	Bl[4]	Bl[3]
Homoarginine	39	R	Pu	Pu	Pu[1]	Y[3]	—	Pu[1]	P-O	—	—
1-Methylhistidine	49	R	Pu-Gy	Pu	Pu[1]	Y[3]	—	Pu[1]	—	—	—
3-Methylhistidine	50	R	Gy-Y	Bl-Gy	P[1]	Y[3]	—	Pu[1]	—	—	—
Ornithine	54	R	Pu	Pu	Pu[1] P[4]	Y[3]	—	P[1] Pu[3]	Bn	—	—
Djenkolic acid	58	Pu	Bn	Pu	R[1]	Y[1,3] Bl[4]	— Y	Bl[1] G[3]	—	—	—
2,6-Diaminopimelic acid	59	R	G	R-Pu	Pu[1]	Y[3]	—	Pu[1]	—	—	—
2,4-Diaminobutyric acid	61	R	Y	Pu	Pu[1] W[3] P[4]	Y[3]	—	Pu[1]	P-O	Y-G[4]	—
Carnosine	75	Bn	Bn	R-Pu	Gy[1] Pu[2] Gy[3] Bn[4]	—	P[1] f	—	—	—	—

In the TLC of amino acids, the low concentration levels (5×10^{-4} μmole) often encountered sometimes affect the response to a particular reagent. For example O-phthalaldehyde (Reagent D) gave a specific green colour with glycine on paper chromatography but a non-specific purple colour on thin layers of cellulose. Nevertheless, most N-containing metabolites can be identified unambiguously by one or

TABLE IV

COLOUR REACTIONS OF METABOLITES OF GROUP II

Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Aspartic acid	3	R	Bl	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Serine	5	R	Gy	P	Pu^{1}	Y^{3}	—	$Bn^{1,3}$	—	Y-G^{4}	—
Glycine	6	Bn	O	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Hydroxyproline	17	Y	Gy	Bl	Bn^{1}	Y^{3}	—	$Pu^{1,3}$	—	—	—
Cysteic acid	20	R	Pu	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
β-Alanine	21	P	Bl	Pu	Pu^{1}	Y^{3}	—	$Bn^{1,3}$	—	—	—
Glutamine	25	R	Gy	R-Pu	Pu^{1}	Y^{3}	f-Y	P^{1}	—	—	—
Phosphoethanolamine	27	R	Pu	Gy-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Taurine	28	R	Gy	P	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
γ-Amino-*n*-butyric acid	30	R	Gy	R-Pu	Pu^{1} P^{4}	Y^{3}	—	Pu^{1}	—	—	—
δ-Aminolaevulinic acid	42	Y	Y	O	Pu^{1}	Y^{3}	—	O^{1} Pu^{3}	—	P^{3}	P^{3}
Methionine sulphone	44	R	Y-G	R-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Methionine sulphoxide	45	R	Y-G	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Sarcosine	51	Pu	Bn	Gy	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
Homoserine	52	R	Pu	P	Pu^{1}	Y^{3}	—	Pu^{1}	—	Y-G^{4}	—
3,4-DOPA	53	Pu	Bn	Bl-Gy	Bn^{1}	Bn^{3} Gy-P^{4}	—	Bn^{1} Gy^{3}	Bn	$Bn^{1,3}$	$Bn^{1,3}$
Citrulline	56	R	Pu	R	Pu^{1}	Y^{3}	Y	Pu^{1} Y^{3}	—	—	—
Penicillamine	57	R	Bl	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	P	Y-G^{4}	—
Formiminoglycine	60	Bn	Y	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	Pu	—	—
5-Hydroxytryptophan	66	R	G	Bl	$Bn^{1,2,3}$ Y^{4}	$Y^{1,3}$ Pu^{4}	Pu	$Bl^{1,3}$	G	$G^{1,3}$ Bn^{2}	$G^{1,3}$ Bn^{2}
Allantoin	76	—	—	—	—	Y^{3}	Y f	Y^{1}	—	—	—

TABLE V

COLOUR REACTIONS OF METABOLITES OF GROUP III

Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Alanine	1	R	Pu	Pu^{1}	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Glutamic acid	4	R	Pu	Pu^{1}	Pu^{1}	Y^{3}	—	Pu^{1}	—	Y-G^{4}	—
Valine	8	R	Pu	R	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
Tyrosine	14	R	Bn	Pu^{1}	Pu^{1}	Y^{3}	—	Bn^{1} Gy^{3}	—	—	—
Proline	16	Y	Y	Bl	Bn^{1}	Y^{3}	—	—	—	—	—
α-Aminoadipic acid	22	R	Pu	Bl-Gy	Pu^{1}	Y^{3}	—	—	—	—	—
β-Aminoisobutyric acid	23	Pu	Y	Bl-Gy	Pu^{1}	Y^{3}	—	Bn^{1}	—	—	—
p-Aminohippuric acid	40	—	—	Y	Pu^{1}	$Y^{1,3}$ Pu^{2} G^{4}	Y	Pu^{1} Y^{3}	Pu	Y-G^{4}	—
α-Aminoisobutyric acid	46	R	—	Gy-P	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
ε-Aminocaproic acid	47	R	Pu	Bl-Gy	Bn^{1}	Y^{3}	—	Pu^{1}	—	—	—
Pipecolic acid	55	Pu	Pu	G	Bl^{1}	Y^{3}	—	Bl^{1}	—	—	—
Saccharopine	64	R	—	P	—	$Pu^{2,4}$	—	—	—	—	—
Hippuric acid	70	—	—	—	—	Pu^{2} Bl^{4}	—	—	—	—	—

TABLE VI

COLOUR REACTIONS OF METABOLITES OF GROUP IV
Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Isoleucine	9	R	Pu	R	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Leucine	10	R	Pu	R	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Phenylalanine	13	R	Bn	Bl-Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Tryptophan	15	R	Gy	Gy-Bl	Pu^{1}	Pu^{2} Y^{3}	Pu	P^{1} Bl-G^{3}	Bl-G	—	—
Methionine	29	R	Gy	R	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Norleucine	32	R	Pu	R	Bn^{1}	Pu^{2} P^{3}	—	Pu^{1}	—	—	—
2-Aminooctanoic acid	33	R	Pu	Pu	Pu^{1}	$Y^{1,3}$	—	Pu^{1}	—	—	—
Ethionine	48	R	—	Pu	Pu^{1} W^{3}	$Y^{1,3}$	—	Pu^{1}	—	—	—
Indican	65	Bl	Pu	Pu	Pu^{1} Y^{2} $Pu^{3,4}$	$Pu^{1,3}$ Y^{2} R^{4}	Pu	$G^{1,3}$	Pu f	$P^{1,3}$ Y^{2} Pu^{4}	P^{1} Y^{2} $Pu^{3,4}$
Tryptamine	68	Gy	Bn	Pu	Gy^{1} $Y^{2,3}$ Pu^{4}	$Y^{1,2}$ Y^{3} Y^{4}	O to Pu	O^{1} Pu^{3}	Bl-G	Y-G^{3} Pu^{4}	f-Y^{3} O^{4}
3-Indolylacetic acid	69	—	Gy-Bn	Y	Bn^{1} $Y^{2,3}$ Bl^{4}	$O^{1,3}$ $Y^{2,4}$	P to Pu-Bn	O^{1}Bl-G Pu^{3}	—	$Y^{1,2,3}$ Bn^{4}	$Y^{1,2,3}$ O^{4}
Urea	71	—	—	—	—	—	Y f	—	—	—	—
Thiourea	74	—	—	—	Pu^{2} Bl^{4}	—	Y f	P^{4}	Bl-Pu	—	$O^{3,4}$

TABLE VII

COLOUR REACTIONS OF METABOLITES OF GROUP V
Conditions and abbreviations as in Table III.

Compound	*No.*	*Colours produced by staining reagents*									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Threonine	7	R	Gy	Pu	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	Bl^{3}
Ethanolamine	26	R	Gy	P	Pu^{1} P^{4}	Y^{3}	—	Bn^{1}	—	Y-G^{4}	—
Cadaverine	35	R	Pu	Bl-Gy	Pu^{1}	Y^{3}	—	Pu^{1}	—	—	—
Putrescine	36	R	Pu	Bl	Bl^{1} W^{3} O^{4}	Y^{1} Y^{3}	Y	Bn^{1} Pu^{3}	P	Y-G^{4}	—
Histamine	37	R	Bn	Bl-Gy	Pu^{1} G^{3}	Y^{3}	—	Pu^{1}	Pu	Y-G^{4}	—
Kynurenine	38	R	O	R	Bl^{1} Bl^{4} Y^{3}	Y^{1} Y^{3} Bl^{2} Bl^{4}	O	Bn^{1} O^{3}	Pu	$Y^{1,3}$ Bl^{4}	$Y^{1,3}$ Bl^{4}
4-Amino-5-imidazole-carboxylic acid	41	—	—	—	Y^{1} Y^{3} Y^{4}	Y^{3} G^{4}	Y	Pu^{1} Y^{3}	Y	O^{1} O^{3} O^{4}	$O^{1,3,4}$
Glucosamine	62	R	Gy	—	Pu^{1}	Y^{3}	—	—	—	—	—
Epinephrine	63	R	Gy	Gy	Bn	Y^{3}	Y	Bn^{1} G^{3}	Y	P^{1} Bn^{3}	P^{1} Bn^{3}
Creatinine	72	—	—	—	Pu^{2} f Pu^{4} f	—	—	—	P	—	—
Guanidine	73	—	—	—	—	—	—	—	P	—	—

TABLE VIII

RESOLUTION OF A MIXTURE OF METABOLITES BY MEANS OF STAINING REAGENTS

Conditions and abbreviations are those indicated in Table III.

Compound	*No.*	*Colours produced by the reagents*					
		A	B	C	D	G	H
Aspartic acid	3	R	Bl	Pu	Pu	Pu[1]	—
Penicillamine	57	R	Bl	Pu	Pu	Pu[1]	P
β-Alanine	21	P	Bl	Pu	Pu	Bn[1,3]	—
Asparagine	24	Bn	Bn	Pu	Pu	Pu[1]	—
Sarcosine	51	Pu	Bn	Gy	Bn	Pu[1]	—
Carnosine	75	Bn	Bn	R-Pu	Gy[1,3] Pu[2], Bn[4]	P[1]	—
3,4-DOPA	53	Pu	Bn	Bl	Bn	Bn[1], Gy[3]	Bn
β-Aminoisobutyric acid	23	Pu	Y	Bl-Gy	Pu	Bn	—
Proline	16	Y	Y	Bl	Pu	Bl[1], Pu[3]	—
δ-Aminolaevulinic acid	42	Y	Y	O	Pu	O[1], Pu[3]	—

TABLE IX

SPECIFIC COLOUR REACTIONS OF SOME METABOLITES

Conditions are those indicated in Table III.

Compound	*No.*	*Reagent*	*Colour reaction*
Glycine	6	B	orange
Histidine	11	D	purple[1], green[3]
Tryptophan	15	G	pink[1], bluegreen[3]
Proline	16	G	blue[1], purple[3]
Hydroxyproline	17	G	purple[1], purple[3]
Glutamine	25	G	pink[1]
Hydroxylysine	31	I	blue[4]
Putrescine	36	D	blue[1], white[3], orange[4]
		G	brown[1], purple[3]
Histamine	37	D	purple[1], green[3]
Kynurenine	38	D	blue[1], yellow[3], blue[4]
		G	brown[1], orange[3]
		I or J	yellow[1], yellow[3], blue[4]
p-Aminohippuric acid	40	E	yellow[1], purple[2], yellow[3], green[4]
4-Amino-5-imidazole-carboxylic acid	41	D	yellow[1], yellow[3], yellow[4]
		I or J	orange[1], orange[3], orange[4]
δ-Aminolaevulinic acid	42	C	orange
		I or J	pink[3]
Ethionine	48	D	purple[1], white[3]
1-Methylhistidine	49	D	pink[1]
3,4-DOPA	53	E	brown[3], grey-pink[4]
		I or J	brown[1], brown[3]
Ornithine	54	G	pink[1], purple[3]
Pipecolic acid	55	C	green
Djenkolic acid	58	G	blue[1], green[3]
2,6-Diaminopimelic acid	59	B	green
2,4-Diaminobutyric acid	61	D	purple[1], white[3], pink[4]
Epinephrine	63	G	brown[1], green[3]

Continued on p. 275.

TABLE IX (*continued*)

SPECIFIC COLOUP REACTIONS OF SOME METABOLITES

Conditions are those indicated in Table III.

Compound	*No.*	*Reagent*	*Colour reaction*
Indican	65	D	purple[1], yellow[2], purple[3], purple[4]
		G	green[1], green[3]
		I	pink[1], yellow[2], pink[3], brown[4]
		J	pink[1], yellow[2], purple[3], purple[4]
5-Hydroxytryptophan	66	D	brown[1], brown[2], brown[3], yellow[4]
		E	yellow[1], yellow[3], purple[4]
		G	blue[1], blue[3]
		I or J	green[1], brown[2], green[3]
Tryptamine	68	D	grey[1], yellow[2], yellow[3], purple[4]
3-Indolylacetic acid	69	I	yellow[1], yellow[2], yellow[3], brown[4]
		J	yellow[1], yellow[2], yellow[3], orange[4]
Carnosine	75	D	grey[1], purple[2], grey[3], brown[4]
Allantoin	76	G	yellow

two of the reagents which we have listed in Table I. Even the most difficult mixture of compounds to analyse such as we have collected together in Table VIII can be distinguished with certainty after TLC by the use of six reagents.

Although it is feasible to identify all of the seventy-six compounds investigated by means of one or more reagents, twenty-seven of them may be identified specifically by one reagent.

This is demonstrated in Table IX where each reagent is capable of producing a unique colour (or fluorescence) sequence when the spot is viewed under successive conditions.

Several well-known specific reagents such as, *e.g.*, the Pauli test for histidine have been omitted from the present work because of their inadequate response at the low levels of concentration which have been used. The fragile nature of the cellulose layer also precludes the use of many other reagents.

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