RAPID PAPER CHROMATOGRAPHIC FRACTIONATION OF COMPLEX MIXTURES OF WATER-SOLUBLE SUBSTANCES

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In research on the constituents of biological extracts our laboratories have often required rapid procedures for rough fractionation and tentative characterization of unknown constituents in small amounts of crude extracts. Paper chromatography on a fast-running paper such as Whatman No. 4 has proved to be the easiest and fastest first stage, and several solvent systems have gradually been evolved for the fractionation of relatively water-soluble substances. A large number of known reference substances have been run in these solvents, and the purpose of this paper is to make available to other workers this accumulation of R_F values.

The first solvent in the series is an 8:8:4:1 mixture of isopropanol, pyridine, water, and glacial acetic acid, for which we commonly use the name IPWA. The use of this solvent for separation of common inorganic ions[1] and of many carbohydrates and polyols[2] has been described previously. A second solvent, BuPWA, a 12:6:4:1 mixture of isobutanol, pyridine, water, and glacial acetic acid, was devised to give lower R_F values and better separation of substances having high R_F values in IPWA[3]. A third solvent, MePWA, a 6:6:4:1 mixture of methanol, pyridine, water, and glacial acetic acid, has not yet been described but is useful for separation of substances having low R_F values in IPWA.

These solvents, used with Whatman No. 4 paper, give complete chromatograms within 2 h and can be sufficiently air-dried even at room temperature in 15 min to allow use of reagents to detect spots. The use of these solvents requires no preliminary equilibration, since their water content is high, and R_F values are not altered by small variations in temperature or solvent composition. The capacity (for substances at least moderately water-soluble) is high, and interference caused by the presence of salts is minimal, since all anions move as the pyridinium salts, and all cations as the acetate salts. Any spray reagent can be used to detect spots, although with certain reagents the chromatograms may require a 24 h drying period or prior extraction with acetone or exposure to steam to remove residual pyridine. We have found that detection of spots by many reagents is improved if the Whatman No. 4 paper is pre-washed before use. The method of GORDON AND HEWEL[1], which uses 80:15:5 distilled water, pyridine, and glacial acetic acid, is satisfactory.

The major disadvantage of these solvents is low resolving power, since relatively

elongated spots are formed, as an unavoidable consequence of the high speed of travel through relatively coarse paper. Since all the solvents are weakly acidic, they do not effect some separations (*e.g.*, arginine from lysine) that are possible in strongly acidic or alkaline solvents in which differential ionization of strongly acidic or basic groups occurs. In fact, our solvents are general-purpose systems and will often be, for any specific separation, inferior to the specialized solvents devised for specific classes of substances. We have used them primarily for the first-stage purification of unknown biologically-active or radioactive compounds; the relatively broad bands so obtained are easily eluted or transferred to a second paper strip for more detailed characterization in a second solvent system or by paper ionophoresis. The original extract is simplified by the preliminary chromatography, and subsequent operations are usually much more informative than they would be if carried out on the crude extracts.

LIMITATIONS OF R_F VALUES IN CHARACTERIZATION OF UNKNOWNS

The compounds for which reference R_F data have been accumulated in our laboratories are mostly of biochemical interest and include many amines and amino acids, peptides, carbohydrates, polyols, carboxylic acids, some dinitrophenyl derivatives, vitamins, antibiotics, nucleosides and inorganic ions. For many of these substances we have previously published ionophoretic mobility data[4,5]. Paper ionophoresis is usually our second stage in identifying unknown substances, because it can yield information about ionizing groups and molecular weight even when the unknown does not match any of our reference compounds.

Table I lists compounds in order of increasing 100 R_F value in BuPWA, IPWA and MePWA. Each listed value is the mean of at least two observed values, usually not differing by more than 3 units, obtained on separate 2-h ascending chromatograms with test spots of 10–20 μg. In our earlier detailed studies using IPWA, however, we have shown that the R_F value and spot size of a substance are affected by the quantity of substance in the initial spot, by its state (*e.g.*, free tartaric acid, potassium tartrate, and calcium tartrate give somewhat different values), and by the kind and amount of other substances present. These effects can be lessened by using pyridine and acetic acid in the original extraction solvent or on the spotted chromatogram to pre-equilibrate the mixture before running, by adding pyridinium sulfate to reduce interference by polyvalent cations or barium acetate to reduce interference by polyvalent anions, and in other ways. Nevertheless, the R_F of a substance in a complex mixture may be 5 or more units higher or lower than that of the pure substance. A substance in a crude extract having a 100 R_F value of 30 may therefore be any one of the reference substances falling in the range from 25 to 35 in the Table; and even larger deviations are sometimes found.

Table I gives values for most of the reference compounds in IPWA and at least one other solvent. The best purification and characterization is achieved in the range from 20 to 50. For example, the best separation of glycine, alanine and α-aminobutyric acid is obtained in IPWA, where the 100 R_F sequence is 20, 36 and 46, while for valine, isoleucine and leucine, BuPWA gives the best sequence (29, 40, 46). Nothing is gained by running a substance with a high IPWA value in MePWA, or a low IPWA value in BuPWA. When values in other solvents are also in the table, these

TABLE I

$100R_F$	*BuPWA*	*IPWA*	*MePWA*
5	DL-Methionine sulfoximine (20, 50)	Adenosine-5′-triphosphate (Na_2) (51) *Djenkolic acid (26) *Lysyl-glycine (42)	
6	Fructose-6-phosphate (Ba) (31, 74) *meso*-Inositol (31, 63) 2-Ketogluconic acid (40, 69) D-Melezitose (54, 78) Scyllo-inosose (38, 68)	Thiamine pyrophosphate (58)	
7	*1,4-Diaminobutane (34, 70) *Glutamic acid (24, 55) *Lactose (46, 73) *Melibiose (45, 74)	*Glycyl-L-histidine (37) *O-Phosphoserine (38) Phosphocholine	
8	DL-Methionine sulfoxide (24, 56) *Potassium ion (15, 45) *Tartaric acid (29, 65)	Ethylene-dinitrilo-tetra-acetic acid (EDTA) (52) *Histidyl-histidine (35)	
9	Gluconic acid (38, 67) Homoserine (31, 60) N-Methyl-glycine (32, 62)	Barium ion (50) Iodate ion (33)	
10	Alanyl-glycine (27, 62) *Alanyl-glycyl-glycine (27, 61) *N-Aminoethyl-piperazine (31, 70) *Calcium ion (35, 75) *Ethylenediamine (28, 61) Glycerophosphoric acid (37, 76) Hydroxyproline (33, 57) Methionine sulfone (37, 63) *Oxalic acid (32, 69) Sarcosine (32, 62) Taurine (38, 61) *Uric acid (45, 70)	Adenosine-5′-diphosphate (Na) (59) *Carnosine (39) Glycyl-asparagine (36) *Homocystine *allo*-Hydroxylysine (44) *Ornithine (46)	
11	*β-Alanine (28, 60) Barbital (diethyl-barbituric acid) (25, 63) *D-Galactosamine (47, 72) *Deoxyadenylic acid (31, 60) *Glycinamide (30, 58)	Ferrocyanide ion *Glycyl-aspartic acid (47)	
12	*Cellobiose (53, 74) Diethylenediamine (32, 66) Gulonic acid (42, 72) *D-Maltose (57, 78) *Phosphate ion (42, 71) Threonine (35, 64)	Asparagine (35) Cysteic acid (39) *Fructose-1,6-diphosphate (Ba) (62)	

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
13	5-Ketogluconic acid (53, 75) *Piperazine (31, 65)	Aminomethylenesulfonic acid (67) *Aspartic acid (40) 2,4-Diaminobutyric acid (41) Ferricyanide ion (72) *Glycyl-glycyl-glycyl-glycine (47) Methionine methylsulfonium ion (47) *O-Phosphoethanolamine (51)	
14	α-Methyl-glutamic acid (38, 73) *Sodium ion (26, 60) *Uridylic acid (49, 75)	*Lysine (49) D-Penicillamine (53) *Spermidine (61)	
15	*α-Alanine (36, 64) *D-Glucosamine (53, 76) *Magnesium ion (57)	*Arginine (51) *Glycyl-glycyl-glycine (52) *Potassium ion (8, 45) Rubidium ion (40) *Tetraethylenepentamine (59)	
16	*Arcaine (41, 78) *Diglycolic acid (54, 74) *Proline (36, 65)	Cesium ion (38) *Histidine (45) Sulfate ion (64)	
17	Arsenate ion (47, 80) *L-Quinic acid (50, 75) *D-Turanose (64, 80)	*Alanyl-asparagine (51) Glycyl-serine (54) Mucic acid (60) Saccharic acid	
18	*3,4-Dihydroxyphenylalanine (42, 66)	Glutamine (45) *Glycyl-L-glutamic acid (55) Glycyl-glycine (47) *2-Thiolhistidine (43)	
19	α-Amino-*n*-butyric acid (46, 74) *FD & C Blue No. 1 (63) *3-Dimethylamino-*n*-propyl-amine (36, 74) Lithium ion (61) *Sucrose (62)	Strontium ion (66)	**meso*-Lanthionine
20	*D-Galactose (62, 78) *Glycyl-L-tyrosine (43, 77) D-Trehalose (52, 75)	*Glycine (51) DL-Methionine sulfoximine (5, 50)	
21	*Calcium ion (35, 75) *Citric acid (62) *Guanine (52, 77) *N-Hydroxyethyl-piperazine (53, 74) Tetramethyl-ammonium ion (47, 81)	*Glucose-6-phosphate (Ba) (69) *Guanylic acid (63)	Glutathione (oxidized)

(continued on p. 48)

TABLE I (*continued*)

$100 R_F$	*BuPWA*	*IPWA*	*MePWA*
22	*D-Glucoheptose (63, 77)		α,ε-Diamino-pimelic acid
23	*L-Cysteinesulfinic acid (48, 71) Glyceric acid (59) Hydantoic acid (49, 72) α-Hydroxyglutaric acid (52, 77)	Glutathione (reduced) (57) *Glycyl-L-proline (58)	
24	Choline (52, 86) *DNP-α,ε-diaminopimelic acid (48, 73) *Dulcitol (galactitol) (64) *Ethanolamine (50, 76) Gluconolactone (62, 90) Glycyl-L-tryptophan (46, 77) *Methylamine (50, 74) Pipecolic acid (48, 75) Quebrachitol (63)	*5′-Adenylic acid (60) Deoxyguanylic acid (62) *Glutamic acid (7, 55) DL-Methionine sulfoxide (8, 56) *Riboflavin-5-phosphoric acid (72)	
25	α-Amino-isobutyric acid (52, 79) Bromate ion (57, 69) *Mannitol (66) *Mucochloric acid (55, 74) Pyridoxamine (58) *D-Sorbitol (64)	Barbital (diethylbarbituric acid) (11, 63) *Sodium ion (14, 60)	
26	Floridoside (65) *D-Glucose (65) *Sedoheptulose (62, 76) Thiamine (70) Tyrosine (56, 77)	*Cytidylic acid (64) *Serine (55)	*Djenkolic acid (5)
27	Acetylcholine (56, 89) Allyl-glycine (49, 74) *2-Amino-2-(hydroxymethyl)-1,3-propanediol (62, 84) *Orotic acid (55)	Alanyl-glycine (10, 62) *Alanyl-glycyl-glycine (10, 61) *Deoxycytidylic acid (65) Lactobionate (68) Vitamin B_{12} (65)	
28	*3-Amino-1-propanol (57) α-Aminovaleric acid (58, 80) Chloride ion (48, 78) 3-Dimethylamino-1,2-propanediol (86) Guanosine (60) Pinitol (67) *2-Pyrrolidone carboxylic acid (56, 75)	*β-Alanine (11, 60) *Ethylenediamine (10, 61) Glycyl-alanine (50)	*allo*-Cystathionine
29	Glycyl-leucine (55, 77) *α-Ketoglutaric acid (66) *Riboflavin (65) Valine (51, 79)	*3′-Adenylic acid (62) *Tartaric acid (8, 65)	

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
30	Dimethylamine (54, 79) *L-Malic acid (63, 81) *D-Mannose (70) Methionine (54, 79) Xanthopterin (48, 68) *Xanthosine (66)	*Glycinamide (11, 58)	
31	*D-Arabinose (65, 79) 2-Dimethylaminoethanol (65) *Fructose (69) Leucyl-glycine (62)	*N-Aminoethylpiperazine (10, 70) *Deoxyadenylic acid (11, 60) Fructose-6-phosphate (Ba) (6, 74) Homoserine (9, 60) *meso*-Inositol (6, 63) *Piperazine (13, 65)	
32	*3-Hydroxy-piperidine (63, 83) *L-Sorbose (69)	*Agmatine (66) Diethylenediamine (13, 66) α-D-Galacturonic acid (67) N-Methyl-glycine (9, 62) *Oxalic acid (10, 69) Sarcosine (10, 62)	Cytidine-diphospho-choline
33	*L-Arabitol (73) Cytidine (60) Guanidine (56, 75) Tryptophan (53, 71)	*Cadaverine (67) Hydroxyproline (10, 57)	Diphosphopyridine-nucleotide Iodate ion (9)
34	*3-Amino-2-propanol (59, 86) *Cytosine (53) Glycyl-phenylalanine (49, 79) *Inosine (67)	*N-Acetyl-L-histidine (62) *1,4-Diaminobutane (7, 70) *Glucose-1-phosphate (Na) (70)	
35	4-Amino-5-imidazolecarboxamide (59, 72) L-Arabinose (70) Bromide ion (66) Deoxyguanosine (79) Isopropylamine (62, 85) Phenylalanine (57, 81) *D-Tagatose (70, 83) *D-Xylose (71)	*Calcium ion (10, 75) *L-Ergothionine (67) Threonine (12, 64)	Asparagine (12) *Histidyl-histidine (8)
36	*Xanthine (64, 76) L-Xylose (73)	*α-Alanine (15, 64) *3-Dimethylamino-*n*-propylamine (19, 74) *Proline (16, 65) Sulfamate ion (60)	Glycyl-asparagine (10)
37	N-Acetylglucosamine (75) *Adonitol (D-Ribitol) (73) Ethionine (61, 81)	*Barbituric acid (55) Cysteine (63) Glycerophosphoric acid (10, 76) *Glycyl-DL-methionine (70) Methionine sulfone (10, 63)	*Glycyl-L-histidine (7) *Glycyl-lysine

(*continued on p. 50*)

TABLE I (*continued*)

$100 R_F$	*BuPWA*	*IPWA*	*MePWA*
38	Cyanoacetic acid (68)	Gluconic acid (9, 67) α-Methyl-glutamic acid (14, 73) Scyllo-inosose (6, 68) Taurine (10, 61)	Cesium ion (16) *O-Phosphoserine (7)
39	ε-Amino-*n*-caproic acid (68, 86) *DNP-*allo*-δ-hydroxylysine (66) *Pyrrolidine (62) *Pyruvic acid (72) *Isoriboflavin (58)		*Carnosine (10) Cysteic acid (12)
40	*5-Hydroxy-2,4-dichloro-phenoxy-acetic acid (75) Isoleucine (63, 82) *D-Lyxose (75) *Malonic acid (71) *Uracil-5-carboxylic acid (61) Urea (62, 75)	5-Hydroxytryptophan (59) 2-Ketogluconic acid (6, 69)	*Aspartic acid (13) Rubidium ion (15)
41	*Acetylglutamic acid (78, 89) *cis*-Aconitic acid (78) Aluminum ion (69) *L-Fucose (75) *p*-Hydroxyphenyl-pyruvic acid (76) Hypoxanthine (62)	*Arcaine (16, 78)	2,4-Diaminobutyric acid (13)
42	*trans*-Aconitic acid (75) Deoxycytidine (69) *Glycolic acid (71) Gulonic lactone (78, 84) Nitro-ferricyanide ion (84) *D-Ribose (76) Tetraethylammonium ion (68, 93)	*3,4-Dihydroxyphenyl-alanine (18, 66) Gulonic acid (12, 72) L-Kynurenine (61) *Phosphate ion (12, 71)	*Lysyl-glycine (5)
43	Adenosine (68)	*Glycyl-L-tyrosine (20, 77)	*allo*-Hydroxylysine (10) *2-Thiolhistidine (18)
44	*Epinephrine (81) *Isocytosine (66, 79)		
45	*Erythritol (77, 85) *Kynurenic acid (63) Nitrate ion (75, 89)	*Melibiose (7, 74) *Raffinose (77) *Uric acid (10, 70)	Glutamine (18) *Histidine (16) *Potassium ion (8, 15)
46	L-Ascorbic acid (85) Chromate ion (70) *3-Hydroxytyramine (76) Leucine (65, 83)	α-Amino-*n*-butyric acid (19, 74) Glycyl-L-tryptophan (24, 77) *Lactose (7, 73) Malto-tetraose	*Ornithine (10)
47	*DNP-djenkolic acid Lead ion (71)	Arsenate ion (17, 80) *D-Galactosamine (11, 72) Isomaltose Tetramethylammonium ion (21, 81)	*Glycyl-aspartic acid (11) Glycyl-glycine (18) Methionine methyl sulfonium ion (13)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
48	*Adenine (60) Chloromycetin (69, 91)	Chloride ion (28, 78) *L-Cysteinesulfinic acid (23, 71) DNP-α,ε-diaminopimelic acid (24, 73) Pipecolic acid (24, 75) Xanthopterin (30, 68)	
49	*DNP-histidine Uridine (84)	Allyl-glycine (27, 74) Glycyl-phenylalanine (34, 79) Hydantoic acid (23, 72) *Uridylic acid (14, 75)	*Lysine (14)
50	*p*-Aminobenzoyl-glutamic acid (80, 89) 2-Deoxy-D-glucose (80) DNP-L-asparagine *Shikimic acid (78) *Xanthurenic acid (74)	*Ethanolamine (24, 76) *Methylamine (24, 74) *L-Quinic acid (17, 75)	Barium ion (9) Glycyl-alanine (28) DL-Methionine sulfoximine (5, 20)
51	Chlorate ion (81) Fumaric acid (80) *Manganous ion (84)	Maltotriose	Adenosine-5′-triphosphate (Na_2) (5) *Arginine (15) *Alanyl-asparagine (17) *Glycine (20) *O-Phosphoethanolamine (13)
52	Deoxyadenosine (70) *L-Rhamnose (82)	α-Aminoisobutyric acid (25, 79) Choline (24, 86) *Guanine (21, 77) α-Hydroxyglutaric acid (23, 77) D-Trehalose (20, 75)	Ethylene-dinitrilo-tetraacetic acid (EDTA) (8) *Glycyl-glycyl-glycine (15)
53		*Cellobiose (12, 74) *Cytosine (34) *D-Glucosamine (15, 76) *N-Hydroxyethyl-piperazine (21, 74) 5-Ketogluconic acid (13, 75) Tryptophan (33, 71)	D-Penicillamine (14) *Glycyl-glycyl-glycyl-glycine (13)
54	DNP-glutamine	*Diglycolic acid (16, 74) Dimethylamine (30, 79) D-Melezitose (6, 78) Methionine (30, 79)	Glycyl-serine (17)
55	Aminotriazole (70) *DNP-glycyl-aspartic acid Iodide ion (78) Thiourea (72, 80) *Tyramine (80)	α-Aminovaleric acid (28, 80) Glycyl-leucine (29, 77) *Mucochloric acid (25, 74) *Orotic acid (27)	*Barbituric acid (37) *Glutamic acid (7, 24) *Glycyl-L-glutamic acid (18) Phosphocholine *Serine (26)
56	Aureomycin (78) Glucuronic lactone (84)	Acetylcholine (27, 89) Guanidine (33, 75) *2-Pyrrolidone-carboxylic acid (28, 75) Tyrosine (26, 77)	DL-Methionine sulfoxide (8, 24)

(continued on p. 52)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
57	Cyclamate ion (83) *Maleic acid (89) Uracil (73)	*3-Amino-1-propanol (28) Bromate ion (25, 69) *Magnesium ion (15) *D-Maltose (12, 78) Phenylalanine (35, 81) Valine (29, 79)	Glutathione (reduced) (23) Hydroxyproline (10, 33)
58	DL-Glyceraldehyde (70) *Leucyl-tyrosine (86, 95)	*Isoriboflavin (39) Pyridoxamine (25)	*Glycinamide (11, 30) *Glycyl-L-proline (23) Thiamine pyrophosphate (6)
59	*Tryptamine (81)	4-Amino-5-imidazolecarboxamide (35, 72) *3-Amino-2-propanol (34, 86) Glyceric acid (23)	Adenosine-5′-diphosphate (Na) (10) 5-Hydroxytryptophan (40) *Tetraethylenepentamine (15)
60	*Lactic acid (82) Picolinic acid (75)	*Adenine (48) Cytidine (33) Guanosine (28)	*5′-Adenylic acid (24) *β-Alanine (11, 28) *Deoxyadenylic acid (11, 31) Homoserine (9, 31) Mucic acid (17) *Sodium ion (14, 25) Sulfamate ion (36)
61	Dihydroxyacetone (86) *DNP-alanyl-glycyl-glycine Glycerol (82) Urocanic acid (80, 86)	Ethionine (37, 81) Lithium ion (19) *Uracil-5-carboxylic acid (40)	*Alanyl-glycyl-glycine (10, 27) *Ethylenediamine (10, 28) L-Kynurenine (42) *Spermidine (14) Taurine (10, 38)
62	*Tricarballylic acid	*2-Amino-2-(hydroxymethyl)-1,3-propanediol (27, 84) *Citric acid (21) *D-Galactose (20, 78) Gluconolactone (24, 90) Hypoxanthine (41) Isopropylamine (35, 85) Leucyl-glycine (31) *Pyrrolidine (39) *Sedoheptulose (26, 76) *Sucrose (19) Urea (40, 75)	*N-Acetyl-L-histidine (34) *3′-Adenylic acid (29) Alanyl-glycine (10, 27) Deoxyguanylic acid (24) *Fructose-1,6-diphosphate (Ba) (12) N-Methyl-glycine (9, 32) Sarcosine (10, 32)
63	*Aesculin (89) *Endophthalic acid (89) Thiocyanate ion (88)	*FD & C Blue No. 1 (19) *D-Glucoheptose (22, 77) *3-Hydroxypiperidine (32, 83) Isoleucine (40, 82) *Kynurenic acid (45) *L-Malic acid (30, 81) Quebrachitol (24)	Barbital (diethyl-barbituric acid) (11, 25) Cysteine (37) *Guanylic acid (21) *meso*-Inositol (6, 31) Methionine sulfone (10, 37)
64		*Dulcitol (galactitol) (24) *D-Sorbitol (25) *D-Turanose (17, 80) *Xanthine (36, 76)	*α-Alanine (15, 36) *Cytidylic acid (26) Sulfate ion (16) Threonine (12, 35)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
65	*DNP-taurine	*D-Arabinose (31, 79) 2-Dimethylaminoethanol (31) Floridoside (26) *D-Glucose (26) Leucine (46, 83) *Riboflavin (29)	*Deoxycytidylic acid (27) *Piperazine (13, 31) *Proline (16, 36) *Tartaric acid (8, 29) Vitamin B_{12} (27)
66	Strychnine (90)	Bromide ion (35) *DNP-*allo*-δ-hydroxylysine (39) *Isocytosine (44, 79) *α-Ketoglutaric acid (29) *Mannitol (25) *Xanthosine (30)	*Agmatine (32) Diethylenediamine (13, 32) *3,4-Dihydroxyphenylalanine (18, 42) Strontium ion (19)
67	*DNP-glycine *2-Phenylethylamine (83)	*Inosine (34) Pinitol (28)	Aminomethylenesulfonic acid (13) *Cadaverine (33) *Ergothionine (35) α-D-Galacturonic acid (32) Gluconic acid (9, 38)
68	*DNP-serine Thymidine (90) Thymine (79)	Adenosine (43) ε-Amino-*n*-caproic acid (39, 86) Cyanoacetic acid (38) Tetraethylammonium ion (42, 93)	Lactobionate (27) Scyllo-inosose (6, 38) Xanthopterin (30, 48)
69	*Flavine-adenine dinucleotide (84)	Aluminum ion (41) Chloromycetin (48, 91) Deoxycytidine (42) *D-Fructose (31) *L-Sorbose (32)	Bromate ion (25, 57) *Glucose-6-phosphate (Ba) (21) 2-Ketogluconic acid (6, 40) *Oxalic acid (10, 32)
70	*DNP-hydroxyproline Hippuric acid (88) α-Ketobutyric acid (92)	Aminotriazole (55) L-Arabinose (35) Chromate ion (46) Deoxyadenosine (52) DL-Glyceraldehyde (58) *D-Mannose (30) *D-Tagatose (35, 83) Thiamine (26)	*N-Aminoethylpiperazine (10, 31) *1,4-Diaminobutane (7, 34) *Glucose-1-phosphate (Na) (34) *Glycyl-DL-methionine (37) *Uric acid (10, 45)
71	6-Chloropicolinic acid (81) Nicotinic acid (83)	*Glycolic acid (42) Lead ion (47) *Malonic acid (40) *D-Xylose (35)	*L-Cysteinesulfinic acid (23, 48) *Phosphate ion (12, 42) Tryptophan (33, 53)
72	Ethylene glycol (86) Nicotinamide (85)	*Pyruvic acid (39) Thiourea (55, 80)	4-Amino-5-imidazolecarboxamide (35, 59) Ferricyanide ion (13) *D-Galactosamine (11, 47) Gulonic acid (12, 42) Hydantoic acid (23, 49) *Riboflavin-5-phosphoric acid (24)

(continued on p. 54)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
73	*Cobalt ion (92) *Nickel ion (92)	*Adonitol (D-Ribitol) (37) *L-Arabitol (33) Uracil (57) L-Xylose (36)	DNP-α,ε-diaminopimelic acid (24, 48) *Lactose (7, 46) α-Methylglutamic acid (14, 38)
74	Phenoxymethyl-penicillin (89) Tetra-*n*-propylammonium ion (82, 94)	*Xanthurenic acid (50)	Allyl-glycine (27, 49) α-Amino-*n*-butyric acid (19, 46) *Cellobiose (12, 53) *Diglycolic acid (16, 54) *3-Dimethylaminopropylamine (19, 36) Fructose-6-phosphate (Ba) (6, 31) *N-Hydroxyethyl-piperazine (21, 53) *Melibiose (7, 45) *Methylamine (24, 50) *Mucochloric acid (25, 55)
75	Beryllium ion (91) Copper ion (88)	N-Acetylglucosamine (37) **trans*-Aconitic acid (42) *L-Fucose (41) *5-Hydroxy-2,4-dichloro-phenoxy-acetic acid (75) *D-Lyxose (40) Nitrate ion (45, 89) Picolinic acid (60)	*Calcium ion (10, 35) Guanidine (33, 56) 5-Ketogluconic acid (13, 53) Pipecolic acid (24, 48) *2-Pyrrolidone-carboxylic acid (28, 56) *L-Quinic acid (17, 50) D-Trehalose (20, 52) Urea (40, 62) *Uridylic acid (14, 49)
76	Gentisic acid (89)	*p*-Hydroxyphenylpyruvic acid (41) *3-Hydroxytyramine (46) *D-Ribose (42)	*Ethanolamine (24, 50) *D-Glucosamine (15, 53) Glycerophosphoric acid (10, 37) *Sedoheptulose (26, 62) *Xanthine (36, 64)
77	*Amphetamine (90) *DNP-threonine	*Erythritol (45, 85)	*D-Glucoheptose (22, 63) Glycyl-leucine (29, 55) Glycyl-L-tryptophan (24, 46) *Glycyl-L-tyrosine (20, 43) Guanine (21, 52) α-Hydroxyglutaric acid (23, 52) *Raffinose (45) Tyrosine (26, 56)
78	Cyclamycin *DNP-glycyl-proline	Acetyl glutamic acid (41, 89) **cis*-Aconitic acid (41) Aureomycin (56) Gulonic lactone (42, 84) Iodide ion (55) *Shikimic acid (50)	*Arcaine (16, 41) Chloride ion (28, 48) *D-Galactose (20, 62) *D-Maltose (12, 57) D-Melizitose (6,54)

TABLE I (*continued*)

$100\ R_F$	*BuPWA*	*IPWA*	*MePWA*
79	Pyridoxine (88)	Deoxyguanosine (35) Thymine (68)	α-Amino-isobutyric acid (25, 52) *D-Arabinose (31, 65) Dimethylamine (30, 54) Glycyl-phenylalanine (34, 49) *Isocytosine (44, 66) Methionine (30, 54) Valine (29, 51)
80	*Itaconic acid (92) Propylene glycol (90) *Succinic acid (93)	*p*-Aminobenzoyl-glutamic acid (50, 89) 2-Deoxy-D-glucose (50) Fumaric acid (51) *Tyramine (55) Urocanic acid (61, 86)	α-Aminovaleric acid (28, 58) Arsenate ion (17, 47) Thiourea (55, 72) *D-Turanose (17, 64)
81	*DNP-α-alanine (95) *DNP-β-alanine	Chlorate ion (51) 6-Chloropicolinic acid (71) *Epinephrine (44) *Tryptamine (57)	Ethionine (37, 61) *L-Malic acid (30, 63) Phenylalanine (35, 57) Tetramethylammonium ion (21, 47)
82	*DNP-glycyl-tyrosine *DNP-proline	Glycerol (61) *Lactic acid (60) *L-Rhamnose (52) Tetra-*n*-propylammonium ion (74, 94)	Isoleucine (40, 63)
83	*2-Chlorophenoxyacetic acid (92)	Cyclamate ion (57) Nicotinic acid (71) *2-Phenylethylamine (67)	*3-Hydroxypiperidine (32, 63) Leucine (46, 65) *D-Tagatose (35, 70)
84		*Flavine-adenine dinucleotide (69) Glucuronic lactone (56) *Manganous ion (51) Nitro-ferricyanide ion (42) Uridine (49)	*2-Amino-2-(hydroxymethyl)-1,3-propanediol (27, 62) Gulonic lactone (42, 78)
85	Cadmium ion (97) *3,4-Dihydroxyphenylacetic acid *DNP-allyl-glycine *DNP-galactosamine *DNP-glycyl-phenylalanine *DNP-methionine *β-Methylglutaconic acid	L-Ascorbic acid (46) Nicotinamide (72)	*Erythritol (45, 77) Isopropylamine (35, 62)
86	*DNP-α-Aminoisobutyric acid *DNP-glucosamine *DNP-phenylalanine Zinc ion (91)	Dihydroxyacetone (61) 3-Dimethylamino-1,2-propanediol (28) Ethylene glycol (72) *Leucyl-tyrosine (58, 95)	ε-Amino-*n*-caproic acid (39, 68) *3-Amino-2-propanol (34, 59) Choline (24, 52) Urocanic acid (61, 80)
87	4-Chloro-3-methyl-phenoxy-acetic acid *DNP-2,4-diaminobutyric acid *DNP-tryptophan		

(*continued on p. 56*)

TABLE I (*continued*)

roo R_F	*BuPWA*	*IPWA*	*MePWA*
88	*Glutaric acid	Copper ion (75) Hippuric acid (70) Pyridoxine (79) Thiocyanate ion (63)	
89	*4-Chloro-2-methyl-phenoxy-acetic acid Hydroxyacetone (97) *2,4,5-Trichlorophenoxyacetic acid	*Aesculine (63) *Endophthalic acid (63) Gentisic acid (76) *Maleic acid (57) Phenoxymethyl-penicillin (74)	Acetyl choline (27, 56) Acetyl-glutamic acid (41, 78) *p*-Aminobenzoylglutamic acid (50, 80) Nitrate ion (45, 75)
90	*p*-Aminobenzoic acid	*Amphetamine (77) Dehydroascorbic acid Propylene glycol (80) Strychnine (66) Thymidine (68)	Gluconolactone (24, 62)
91	*Gibberellic acid Quinine	Beryllium ion (75) Zinc ion (86)	Chloromycetin (48, 69)
92	*DNP-alanyl-leucine *DNP-α-aminobutyric acid *DNP-methionine sulfoximine *2-Hydroxyphenylacetic acid	*2-Chlorophenoxyacetic acid (83) *Cobalt ion (73) *Itaconic acid (80) α-Ketobutyric acid (70) *Nickel ion (73)	
93	Caffeic acid	*Succinic acid (80)	Tetraethylammonium ion (42, 68)
94			Tetra-*n*-propylammonium ion (74, 82)
95	*DNP-isoleucine DNP-leucine *DNP-methionine sulfoxide *Indole-3-acetic acid	*DNP-α-alanine (81)	*Leucyl-tyrosine (58, 86)
96-97	*Apolon *DNP-α-aminobutyric acid *2-Hydroxycinnamic acid *4-Hydroxycinnamic acid *2-Hydroxy-4-methoxycinnamic acid *Umbelliferone (7-hydroxy-coumarin)	Cadmium ion (85) Hydroxyacetone (89)	

are given in parentheses following the name of the compound at each place where it is listed; when ionophoretic mobility values have been published for the compound, its name is preceded by an asterisk. Thus, in the BuPWA 15 column, one finds *alpha-alanine (36, 64) to indicate that this compound is listed (together with compounds that have the same value) in the IPWA 36 and MePWA 64 columns and also in the ionophoretic tables of THORNBURG *et al.*[5]. Values in a second and third solvent

may support a tentative identification but since these solvents differ in "moving power" rather than in resolving power, they are not sufficient for final characterization of an unknown.

USE OF THE CHROMATOGRAPHIC SOLVENTS

The extract of an unknown material may be made in water, but we normally use 10% or 20% methanol containing 1% of pyridine and 1% of acetic acid to effect a pre-equilibration, and also to permit storage without decomposition by microorganisms. If the unknowns are thermo-stable, briefly heating in boiling water is desirable to coagulate proteins, which may otherwise ruin the chromatographic separation. In many instances 50% methanol can be used to give an extract relatively free of proteins and of excessive amounts of salts, sugars, or other major constituents. After spotting and drying, brief extraction of the spot with acetone can sometimes eliminate acetone-soluble undesirable impurities. Extracts or spotted strips can be stored in a freezer below 0°C, but oxidative or hydrolytic decomposition can be a serious problem, which can only be solved by lyophilizing in glass ampoules, vacuum-sealing, and storing at low temperature.

Our simple apparatus for one-dimensional ascending chromatography has been described[1] and is commercially available in somewhat modified form from Microchemical Specialities Company, Berkeley, Calif.

All extracts are first run in IPWA, with several test spots to allow use of different reagents to locate acidic or basic spots, polyols, amines, fluorescent substances, or other classes of compounds. With extracts containing radioactive substances, one strip is scanned with a thin mica window Geiger counter to locate spots of high activity. When biological activity (vitamins, antibiotics) is being investigated, strips are cut into regions (usually 0–30, 30–70, and 70–100) which are eluted and tested. A second test strip is subdivided to test the boundaries (*e.g.*, 20–40, or 60–80). The results usually make possible localization within a band not more than 20 units in width.

When a substance has a value less than 40 in IPWA, the extract is also run in MePWA; when the IPWA value is higher than 60, the extract is also run in BuPWA.

When an unknown has been located, a large quantity of extract is banded on a relatively wide strip of washed paper and run in the solvent in which its 100 R_F value is nearest to 40. The region containing the desired band is cut out of this preparative strip. Usually the cut is narrow and designed to remove the diffuse upper and lower regions of the band. While this causes some loss, the bulk of the material is obtained in purer form by such trimming. The cut-out strip can be eluted with several ml of water or 10% methanol, but the solution so obtained may be too dilute for subsequent work. Such elution is usually done only when a degradation or derivatization (such as reaction with dinitrofluorobenzene[5]) is to be attempted, or passage through an ion-exchange column is planned. For direct transfer to a second paper strip, we usually insert one edge of the strip cut from the chromatogram in water and let the capillary flow move the solutes to the other edge. When this edge is touched briefly to a point or line on a strip of paper to be used for further chromatography or for paper ionophoresis, the solutes are transferred as a relatively concentrated solution. To check the efficiency of transfer, a spot of a dilute dye solution can be applied to the center of

the strip before it is dipped in water. When paper ionophoresis by the method of WERUM *et al.*[4] is to be used, a spot of the reference dye solution ABA will serve both as an indicator of transfer and a mobility standard in the ionophoresis.

USE OF OTHER PAPERS AND DEVELOPMENT INTERVALS

When Whatman No. 4 paper is used, development intervals longer than 2 h do not much improve separation of spots. Shorter periods (as little as 10 min) are occasionally useful when a desired substance has an R_F much higher or lower than the substances from which it is to be separated. For example, serine (IPWA 26) and glycerol (IPWA 82) are well separated in 10 min. Since spots in such short chromatograms are more compact, smaller quantities can be detected. Similar compact chromatograms can be produced by using slower papers (such as the medium-speed S & S 2043-B, or the very slow S & S 589) for 2- or 3-h runs. We use 2-h runs on S & S 2043-B when minor spots are difficult to detect in the Whatman No. 4 strips, since spot area on slow papers is usually not more than twice that of the initial spot (while on Whatman No. 4 strips the area may be 10 times larger, largely because of outward diffusion in all directions). Longer runs (4–8 h) on S & S 2043-B are sometimes useful when maximal resolving power is required, since the compactness of spots often allows complete separation of substances with small R_F differences. For small-scale isolation, as described in the preceding section, we still prefer full-length chromatograms on Whatman No. 4 paper because the desired bands can be cut out with less attention to the precise location of the band. On small compact strips an error of a millimeter or two in the location of the cuts can greatly decrease the yield or the purity of the isolated band. Thicker papers (such as Whatman 3 MM or S & S 470) increase the capacity of chromatograms, but usually with a loss either in speed or in resolution of spots.

COMPARISON OF REFERENCE R_F VALUES WITH THOSE IN OTHER SOLVENTS

For carbohydrates, it has been shown that the sequence of R_F values is the same in most solvents, except phenol–water[6]. This makes it possible to estimate values for carbohydrates not included in Table I, by comparison with published data of other workers. For example, KAISER[7] has presented data for rhamnose (BuPWA 52) and several exotic sugars found in cardiac glycosides, from which it can be estimated that digitalose would fall near BuPWA 60 and digitoxose near BuPWA 75.

Unfortunately many other compounds are much more sensitive to the composition of the chromatographic solvent. For example, the R_F sequence for alanine, valine, pyroglutamic acid, and leucine in collidine–lutidine solvent[8,9] is similar to that in IPWA, but taurine has an R_F near that of valine while in IPWA it is near alanine. In 9:1 *n*-butanol–acetic acid/water, the R_F sequence of taurine, alanine, valine and leucine[9] is similar to that in BuPWA, but pyroglutamic is near leucine while in BuPWA it is near valine. R_F values even in solvents somewhat similar in composition to ours are not useful for prediction of R_F values in the IPWA group of solvents, except possibly when two or more reference R_F values in a homologous series in another solvent can be fairly closely matched with values in one of the IPWA group.

The IPWA group is the result of selection from several hundred experimental solvent systems. Many of the systems tried were variations in proportions of the same

components, which gave slightly different R_F values but fundamentally similar chromatograms. Other components (such as 2-butanone, acetonitrile, propionic acid) were occasionally tried but never produced clearly superior chromatograms. BuPWA, IPWA and MePWA were finally adopted in order to have a minimal number of standard systems, different primarily in "moving power". Many attempts to attain a general-purpose one-phase system with much lower "moving power" than BuPWA have been unsuccessful. Many specialized systems containing relatively hydrophobic liquids such as benzene or benzyl alcohol are described in the literature, but in our experience some of these have proved to be of low capacity and very sensitive to interference, to give erratic R_F values, or to be incompatible with many spray reagents.

Of the many solvents described in the literature that differ considerably in resolving power from the IPWA group, we have found an 8:8:4:1 mixture of *tert.*-butanol, methyl ethyl ketone, water and diethylamine most useful for rapid paper chromatography. This is essentially the alkaline system used by REDFIELD[10] in small-scale two-dimensional paper chromatography. It is more sensitive to salts and other interfering factors than the IPWA group and not as useful as a first solvent for crude extracts.

SUMMARY

Three alcohol–pyridine–water–acetic acid solvent systems have proved useful for preliminary fractionation of crude biological extracts by paper chromatography in 2 h or less. High solute concentrations and the presence of salts are usually tolerated but proteins may interfere. The primary system contains isopropanol; this is replaced by isobutanol if R_F values of substances to be isolated are too high for optimal resolution, and by methanol if R_F values are too low. R_F data for many organic and inorganic compounds in the three systems are tabulated. Unknown substances are recovered from the chromatograms in partially purified form for further characterization by other chromatographic solvents or paper ionophoresis.

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