A VERSATILE SOLVENT TO REPLACE PHENOL FOR THE PAPER CHROMATOGRAPHY OF RADIOACTIVE INTERMEDIARY METABOLITES

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Analysis of the products of radioactive tracer feeding experiments in biological systems has depended to a considerable extent on paper chromatography. Of the many solvent systems proposed, the one devised by BENSON *et al.*[1], based on the use of phenol–water followed by *n*-butanol–propionic acid–water, has many advantages for the separation of such biological intermediates as sugars, sugar phosphates, amino acids and carboxylic acids. Nevertheless, phenol–water mixtures have several inherent drawbacks; MIZELL AND SIMPSON[2] recently surveyed some of these, and to this list we might add both the corrosive action of phenol on human skin and the fact that many amino acids show signs of decomposition when chromatographed in phenol-containing solvents[3,4].

The quest for a solvent to replace phenol led us to consider some of those mentioned in the literature[2,5,6], but none were suitable for our purpose. Eventually interest centered on solvents based on isobutyric acid, ammonia, water and ethylenediaminetetraacetic acid (EDTA)[7]. Although this solvent was not entirely satisfactory as originally formulated[7], because such slow-running hydrophilic substances as sugar phosphates migrated too far and left empty much of the chromatogram near the origin, the mixture seemed promising as a basis for further experimentation. There were two approaches towards improving both resolution and the spread of compounds along the whole length of the chromatogram: manipulation of the pH, and an enhancement of the non-aqueous character of the liquid. The pH of the original isobutyric acid–ammonia–water–EDTA solvent is about 4.2; replacement of some of the water with sufficient ammonia solution to raise the pH to 6.8 resulted in a relatively viscous and very slow-running solvent in which the resolution and spread actually deteriorated.

The first attempts to reduce the aqueous nature of the solvent consisted of replacing the ammonia solution, partially or completely, with such organic bases as pyridine and trimethylamine in various combinations, always maintaining the pH at 4.2. A further variation was to saturate the original solvent mixture with toluene. It soon became apparent that the use of these substances did little to improve the chromatographic separation; however, we were stimulated to invent quite a complex vocabulary to describe the olfactory impact of the products. Subsequent alteration of the solvent mixtures was directed to a partial replacement of the water by one of

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a number of simple alcohols: ethanol, *n*-propanol, isopropanol and *n*-butanol. This led to marked improvements in solvent characteristics, though no single alcohol was completely satisfactory in this regard. Mixing of those alcohols which looked promising when used singly was then attempted. After numerous trials, a mixture (hereafter designated "'semi-stench'") was prepared which possessed the most suitable solvent properties for our purposes.

EDTA	1.2 g
17 *N* Ammonia solution	100 ml
Water	950 ml
n-Propanol	350 ml
Isopropanol	75 ml
n-Butanol	75 ml
Isobutyric acid	2500 ml

On standing at room temperature for 24 h esterification equilibria appeared to be established between the alcohols and isobutyric acid. The ammonium isobutyrate buffered the mixture at a pH of about 4.0. Aging of the mixture, even for a period of weeks, effected no discernible change in resolution properties. On the other hand, a comparison of mixtures aged for at least 24 h with corresponding solutions freshly prepared showed that aging for one day is mandatory for optimal results. No attempt was made to accelerate esterification either by mild heating or by refluxing, since it was quite convenient to prepare the solvent at least a day before use.

For chromatographic development, the two-dimensional descending technique was employed. The semi-stench solvent was used for the first dimension; the second dimension was developed with *n*-butanol–propionic acid–water[11]. Standard Whatman No. 4 chromatography paper was used exclusively; neither oxalic acid-washed paper nor paper rinsed in an alkaline EDTA solution (pH 8.5) was more effective. Both solvents were allowed to run to the edges of the paper, this taking (23°) 10–14 h for the first dimension (*ca.* 50 cm) and 7–10 h for the second (*ca.* 38 cm), depending mainly on the batch of paper. Known compounds were localized by spraying or dipping the paper in the appropriate reagents. Radioactive substances were found by radioautography, positive identification then being made by cochromatography with authentic marker compounds.

In our actual experimental conditions, the compounds of interest are chromatographed in the presence of cell extracts. Since there is probably interaction between compounds in mixtures, the chromatographic parameters of all the compounds investigated were measured by mixing each authentic substance with an ethanol–water extract of *Chlorella pyrenoïdosa* cells or of spinach chloroplasts which had been allowed to fix $^{14}CO_2$ photosynthetically[1]. Table II lists the relative R_F values for all the compounds studied with respect to aspartic acid.

The mobilities reported in Table I must be used only as an approximate guide to chromatographic position when compounds are being studied in a complex mixture. These values were obtained by adding two or three known substances at a time to the ^{14}C-labelled plant extract, and relating their position to aspartic acid. We cannot therefore be certain that compounds having nearly identical mobilities will have the precise relationships to each other indicated in Table II, since we have not simultaneously chromatographed all the listed compounds on one sheet of paper. Slight variations between different chromatograms in a series preclude absolute certainty

TABLE I

$R_{aspartic\ acid}$ VALUES IN SEMI-STENCH (A) AND IN *n*-BUTANOL–PROPIONIC ACID–WATER (B)

Compound	$R_{aspartic\ acid}$	
	A	*B*
Amino acids and peptides		
α-Alanine	2.49	1.30
β-Alanine	2.65	1.66
γ-Aminobutyric acid	3.46	2.04
Arginine	2.90	1.14
Asparagine	1.50	1.00
Aspartic acid	1.00	1.00
Citrulline	1.85	1.13
Cysteine	1.01	0.63
Cystine	0.81	0.63
Glutamic acid	1.25	1.08
Glutamine	1.58	0.92
Glutathione, oxid.	0.10	0.55
Glutathione, red.	1.07	0.46
Glycine	1.82	0.96
Histidine	3.66	0.42
Leucine	3.92	2.30
Isoleucine	3.50	2.77
Lysine	2.82	0.67
Methionine	3.23	2.33
Phenylalanine	3.62	2.55
Proline	3.07	1.65
Serine	1.70	0.94
Threonine	2.22	1.20
Tryptophan	2.68	1.77
Tyrosine	2.36	1.64
Valine	3.44	2.61
Carboxylic acids, hydroxy- and oxo-carboxylic acids		
cis-Aconitic acid	0.89	1.48
cis-Aconitic anhydride	0.87	1.76
Citric acid	0.79	1.46
Isocitric acid	0.84	1.57
Fumaric acid	1.13	1.83
Glyceric acid	1.43	1.52
Glycollic acid	1.77	1.73
Glyoxylic acid	1.83	1.55
Lactic acid	2.47	2.16
Malic acid	1.09	1.58
α-Oxoglutaric acid	1.12	1.51
Pyruvic acid	1.75	1.60
Succinic acid	2.00	1.87
Tartaric acid	0.53	0.94
Sugars		
Arabinose	1.53	1.26
2-Desoxyribose	2.65	1.96
Dihydroxyacetone	2.00	2.11
Erythrose	1.71	1.32
Erythrulose	2.19	1.67
Fructose	1.52	1.15
Galactose	1.18	1.02
Glucose	1.20	1.05

(continued on p. 222)

TABLE I (*continued*)

Compound	$R_{aspartic\ acid}$	
	A	*B*
Gulose	1.30	1.07
Lactose	0.63	0.64
Lyxose	1.59	1.29
Maltose	0.74	0.74
Mannose	1.17	1.01
Ribose	1.83	1.36
Ribulose	2.13	1.43
Sedoheptulose	1.21	1.07
Sorbose	1.38	1.13
Sucrose	1.26	0.99
Threose	2.28	1.67
Xylose	1.60	1.34
Xylulose	1.95	1.29
Sugar alcohols		
Arabitol	1.75	1.19
Dulcitol	1.47	1.06
Glycerol	3.03	1.91
Mannitol	1.55	1.14
Ribitol	1.53	1.20
Sorbitol	1.53	1.12
Xylitol	1.72	1.15
Uronic acids		
Glucuronic acid	0.72	0.77
Glucuronolactone	1.67	1.37
Aldonic acids		
Ascorbic acid	1.42	1.69
Gluconic acid	0.78	1.04
Nucleosides		
Adenosine	3.51	1.98
Cytidine	2.54	1.66
Guanosine	1.50	1.37
Inosine	1.42	1.24
Thymidine	2.81	2.47
Uridine	1.67	1.38
Nucleotides		
Adenosine monophosphate	1.35	0.86
Adenosine diphosphate	0.68	0.35
Adenosine triphosphate	0.37	0.23
Guanosine monophosphate	1.35	0.86
Guanosine diphosphate	0.21	0.39
Guanosine triphosphate	0.08	0.22
Inosine monophosphate	0.59	0.49
Thymidine monophosphate	1.27	1.06
Uridine monophosphate	0.91	0.79
Uridine triphosphate	0.10	0.21
Sugar monophosphates		
Dihydroxyacetone phosphate	0.81	0.77
Fructose-6-phosphate	0.55	0.65

TABLE I (*continued*)

Compounds	$R_{aspartic\ acid}$	
	A	*B*
Glucose-6-phosphate	0.42	0.57
Hamamelose-6-phosphate	0.51	0.73
Maltose monophosphate	0.35	0.47
Ribose-5-phosphate	0.68	0.75
Sedoheptulose-7-phosphate	0.42	0.57
Sugar diphosphates		
Fructose diphosphate	0.14	0.24
Hamamelose diphosphate	0.14	0.35
Ribulose diphosphate	0.22	0.39
Miscellaneous phosphates		
Phosphoenolpyruvic acid	0.71	1.16
6-Phosphogluconic acid	0.23	0.71
2-Phosphoglyceric acid	0.60	0.90
3-Phosphoglyceric acid	0.53	0.88
Uridine diphosphoglucose	0.44	0.31

of chromatographic mobility for any particular substance. The mobilities in two dimensions of over twenty compounds was measured with respect to aspartic acid on eight replicate chromatograms similar to the one shown in Fig. 1. The standard deviation of these measurements showed an average for all the spots of $\pm$ 2.8% of the means in the semi-stench solvent, and $\pm$ 3.9% of the means in *n*-butanol–propionic acid–water. Thus the values reported in Table I should be regarded as possessing an error of *ca.* 6–8%.

A radioautogram of a typical chromatogram of ^{14}C-labelled chloroplast extract is shown in Fig. 1, compared with a parallel chromatogram of a similar extract run in phenol–water[1] as the first solvent (Fig. 2). It will be seen that with the semi-stench solvent there is a more effective use than with phenol–water of the total area available on the chromatogram. Both systems have disadvantages resulting from the overlapping of certain compounds which it would be most desirable to have separated. In semi-stench plus butanol–propionic acid this affects mainly glutamine, glycine and serine, while in phenol plus butanol–propionic acid, glutamine runs well clear of the other two amino acids though the latter cannot be separated from glucose. The new system, moreover, results in spots more compact than does phenol, and shows much less variability as far as diffuseness and streaking of the spots is concerned.

In comparing these two solvent systems one effect has consistently been observed, but has not been completely resolved. Using the same cell extract, more spots are separated in the semi-stench than in the phenol system. We think that these are neither artifacts nor degradation products, but reflect the greater resolving power of semi-stench. A preliminary study of the action of semi-stench as far as the decomposition of amino acids is concerned has shown it to be much milder than phenol: the latter causes considerable breakdown of some amino acids[3,4]. The reason for this destruction by phenol is possibly a result of atmospheric oxidation of phenol to form

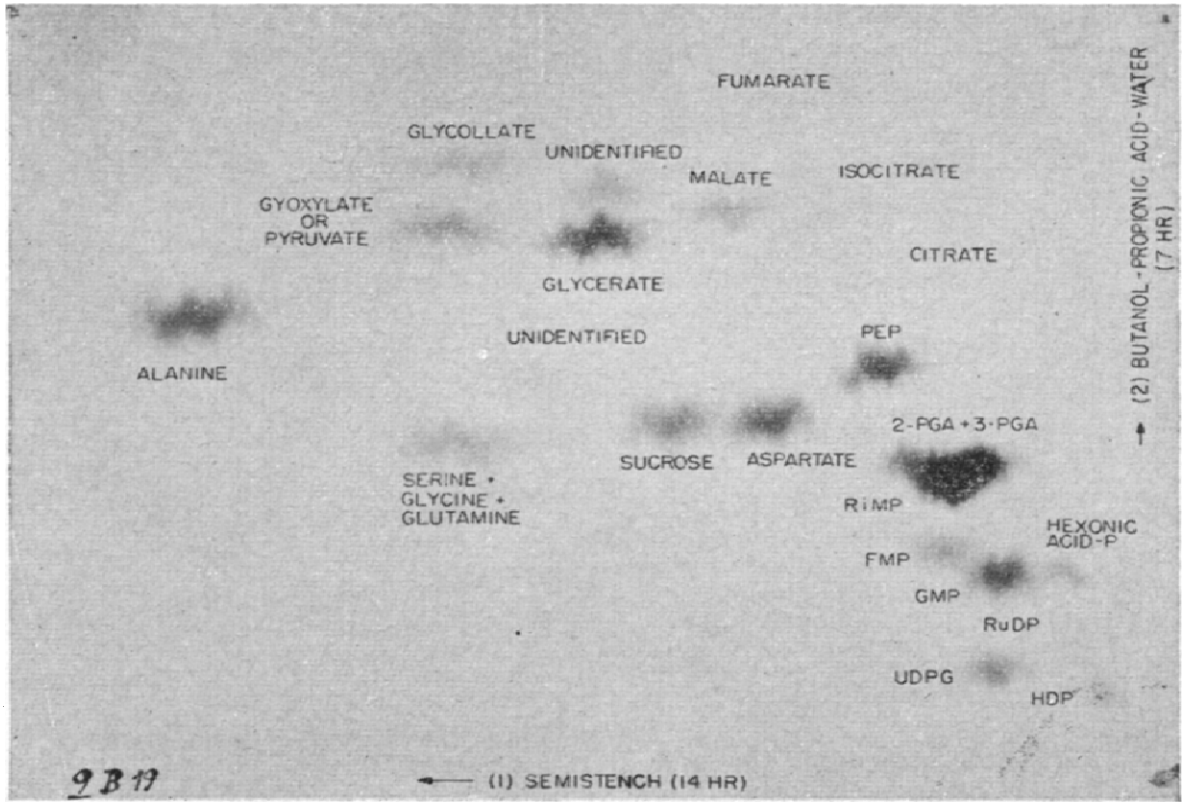

Fig. 1. Radioautograph of chromatogram of extract of ^{14}C-labelled chloroplasts, developed to the edges of the paper only with (1) semi-stench (14 h) and (2) *n*-butanol–propionic acid–water (7 h). FMP = fructose monophosphate; GMP = glucose monophosphate; HDP = glucose and fructose diphosphates; hexonic acid-P = monophosphate of an unidentified hexonic acid; PEP = phoshoenolpyruvic acid; 2-PGA = 2-phosphoglyceric acid; 3-PGA = 3-phosphoglyceric acid; RiMP = ribose monophosphate; RuDP = ribulose diphosphate; UDPG = uridinediphosphoglucose.

the deep red phenoquinone, a relatively strong oxidizing agent. None of the preservatives generally recommended for phenol chromatography completely inhibits the process, though the addition of α-tocopherol or potassium cyanide to the phenol–water mixtures does slow down oxidation[8].

It must be pointed out that some concern may justly be accorded those substances (acid anhydrides, enolic esters, and other high energy compounds) which are often more susceptible to ammonolysis than to hydrolysis. It is possible that some of the newly differentiated spots may be products of ammonolysis of certain of the groups mentioned. While we have neither proven nor disproven this, it should be noted that the versatility of the new solvent system is such that an equimolar quantity of trimethylamine may be substituted for the ammonia without affecting the chromatographic pattern[9].

As with the phenol system, chromatograms developed only to the edges of the paper with semi-stench and butanol–propionic acid show the phosphate esters congregated close to the origin and inadequately separated. Better resolution of these substances may be obtained by over-running the chromatograms for 40–60 h in semi-stench and 20–24 h in butanol–propionic acid. However, in this case, the mobilities of the various phosphates with respect to aspartic acid are not the same as when the chromatograms are developed to the edges of the paper only. Table II lists the

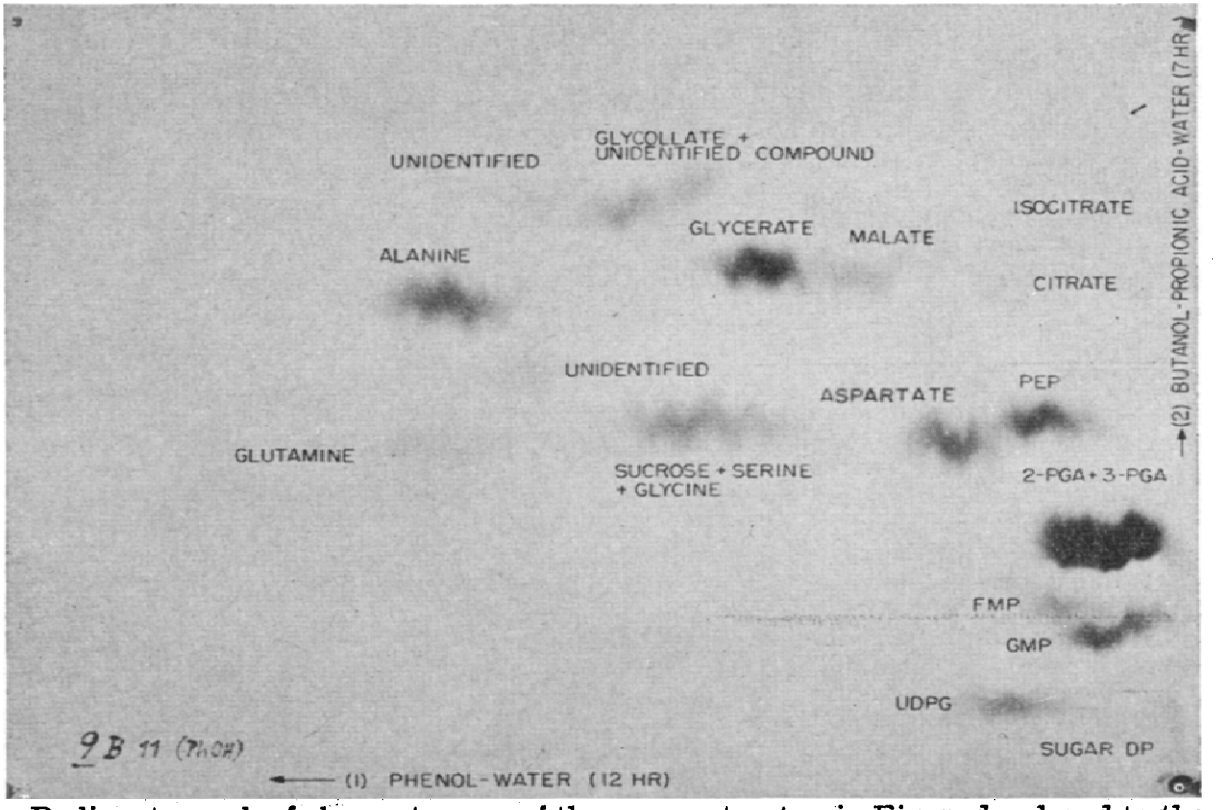

Fig. 2. Radioautograph of chromatogram of the same extract as in Fig. 1, developed to the edges of the paper only with (1) phenol–water (12 h) and (2) *n*-butanol–propionic acid–water (7 h). Sugar DP = sugar diphosphates; other abbreviations as for Fig. 1.

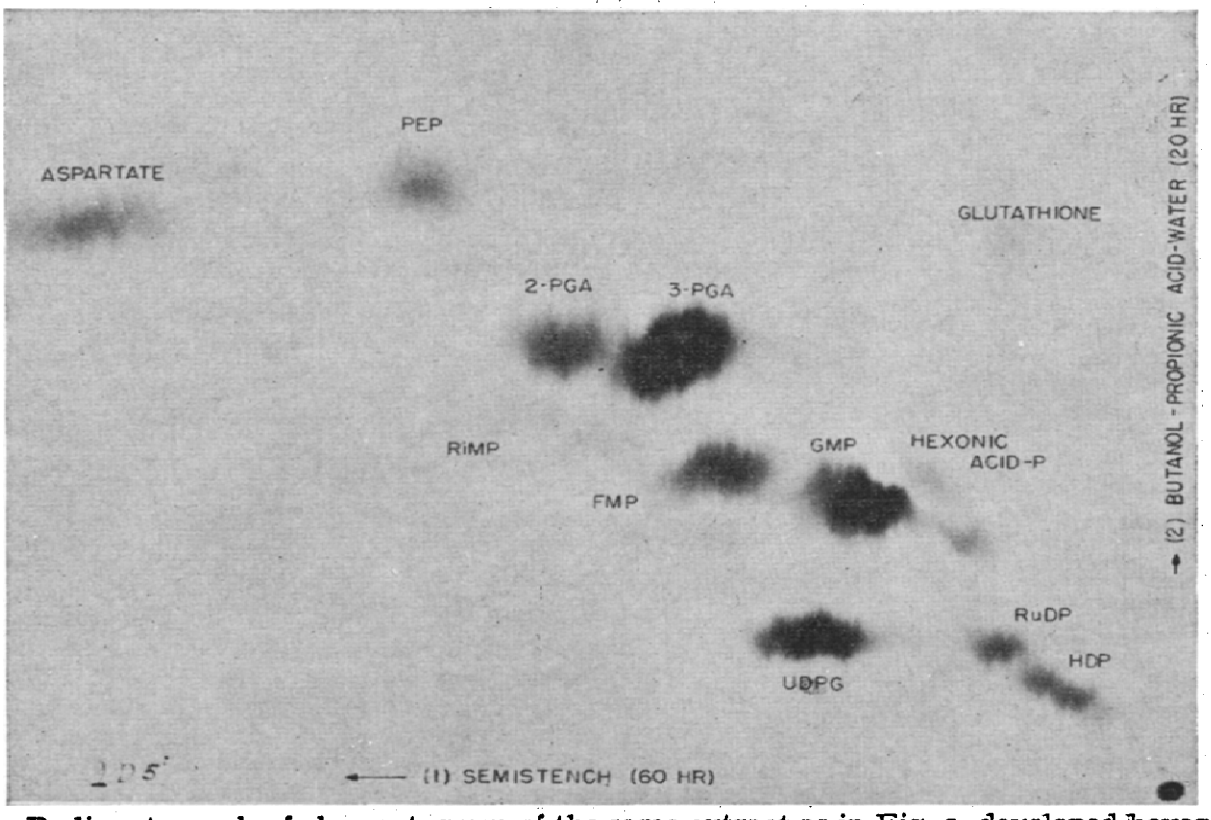

Fig. 3. Radioautograph of chromatogram of the same extract as in Fig. 1, developed beyond the edges of the paper with (1) semi-stench (60 h) and (2) *n*-butanol–propionic acid–water (20 h). Abbreviations as for Fig. 1.

relative R_F values under over-running conditions with reference to 3-phosphoglyceric acid, and a radioautogram is shown in Fig. 3. These different mobilities after prolonged chromatographic development may probably be ascribed to three factors: (i) greater esterification in the solvent at increasing times after mixing: this would be

TABLE II

EFFECT OF OVER-RUNNING ON $R_{3\text{-phosphoglyceric acid}}$ OF PHOSPHATES

Chromatograms developed either to edge of paper (12 h for semi-stench; 8 h for *n*-butanol–propionic acid–water) or over-run for 60 h in semi-stench and 20 h in *n*-butanol–propionic acid–water (BuPr).

Compound	$R_{3\text{-phosphoglyceric acid}}$			
	Chromatogram developed to edge of paper only		Chromatogram over-run	
	Semi-stench	BuPr	Semi-stench	BuPr
Fructose-6-phosphate	1.03	0.75	0.91	0.73
Fructose diphosphate	0.27	0.29	0.23	0.23
Glucose-6-phosphate	0.77	0.68	0.63	0.66
Phosphoenolpyruvic acid	1.33	1.36	1.53	1.38
2-Phosphoglyceric acid	1.00	1.00	1.24	1.02
3-Phosphoglyceric acid	1.00	1.00	1.00	1.00
Ribose-5-phosphate	1.28	0.88	1.13	0.79
Ribulose diphosphate	0.41	0.46	0.35	0.33
Uridinediphosphoglucose	0.83	0.37	0.74	0.35
Aspartic acid	1.88	1.17	2.22	1.27

TABLE III

ALTERATION OF SOLVENT COMPOSITION DURING CHROMATOGRAM DEVELOPMENT

Percentage (v/v) of each component in the mixture

	Water	Isopropanol	n-Propanol	n-Butanol	Isobutyric acid
1. Semii-stench					
Stock bottle	26.5	1.9	9.1	1.9	60.6
Chromatography trough, after development	26.2	1.8	8.5	3.2	60.4
Chromatogram, 0–7.5 cm	18.4	1.0	6.3	7.7	66.5
Chromatogram, 7.5–15 cm	16.6	1.1	6.0	7.7	68.7
Chromatogram, 15–22.5 cm	15.5	1.2	6.3	7.5	69.6
Chromatogram, 22.5–30 cm	16.5	1.2	6.6	7.4	68.3
Chromatogram, 30–37.5 cm	16.0	1.4	6.6	7.5	68.4

	Water	n-Butanol	Propionic acid
2. n-Butanol–propionic acid–water			
Stock bottle, fresh	30.7	46.8	22.5
Stock bottle, after 24 h	31.5	49.0	19.5
Chromatography trough, after development ((24 h old))	25.7	50.1	24.2
Chromatogram, 0–7.5 cm	22.1	50.4	27.6
Chromatogram, 7.5–15 cm	14.9	55.4	29.6
Chromatogram, 15–22.5 cm	15.7	56.1	28.2
Chromatogram, 22.5–30 cm	16.3	57.5	26.2
Chromatogram, 30–37.5 cm	12.9	58.1	29.0

of greater significance with butanol-propionic acid–water, as this solvent is freshly prepared and esterification would thus be progressive; (ii) differential evaporation of component substances in the mixture; (iii) paper chromatographic separation of the solvent constituents themselves, resulting in a series of bands of varying composition along the chromatogram.

Differences in solvent composition at varying stages after preparation and during one-dimensional chromatograms are shown in Table III. After development the wet chromatogram was cut into strips, 7.5 cm wide, perpendicular to the direction of solvent travel. Liquid was obtained from each strip, as well as from residual solvent in the trough. All these samples were analyzed by vapour phase chromatography, using a polymetaphenyl ether column at 90° flushed with helium. Quantitative determinations of the separated components were made by measurements of the peak heights. Small amounts of esters would probably have been obscured by the carboxylic acid peak. It will be seen from Table III that *n*-butanol–propionic acid–water, but not semi-stench, loses water simply by standing in the trough. Both solvents show changes as they flow down the paper, becoming relatively poor in water with increasing distance travelled. Semi-stench also becomes relatively poorer in *n*-propanol and isopropanol and both solvents become richer in *n*-butanol and in the acid component.

Some substances, notably carboxylic acids, have different mobilities in butanol–propionic acid, depending on whether the solvent in the first dimension was semi-stench or phenol–water. This might be explained as a result of the formation of the ammonium salts after development in semi-stench, the ammonium salts then having lower mobilities than the free acids in butanol–propionic acid. This would be particularly significant for the dicarboxylic and oxocarboxylic acids, all of which are considerably stronger acids than propionic.

Two further properties of semi-stench are worthy of mention. Firstly, the flexibility of the system enables its hydrophilicity to be increased or decreased at will to suit particular circumstances. Increasing the water content at the expense of the alcohols allows the more water-soluble components to migrate further, and *vice versa*. The second property is of value in preparing substances for use in microbiological assay and in enzymology. No matter for how long a paper chromatogram is dried it is almost impossible to remove all traces of many solvents, even after development with another solvent. Trace amounts of phenol can poison both enzymic and microbiological systems, while traces of isobutyric acid pose no apparent difficulty in this respect.

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SUMMARY

A new solvent system, to avoid the use of phenol–water, has been developed for the chromatography of complex mixtures of intermediary metabolites. The solvent contains ethylenediaminetetraacetic acid, ammonia, water, isobutyric acid and four simple aliphatic alcohols, and is stable at least for many weeks. The advantages and properties of this solvent are discussed, and the mobilities in it of about one hundred metabolic intermediates are listed with reference to the mobility of aspartic acid.

Note added in proof

It has been found that the chromatographic resolution and the sharpness of the spots are greatly improved by the use of papers other than Whatman No. 4. Whatman No. 2 is distinctly superior to Whatman No. 4, but the best results have been obtained with Ederol No. 202 (J. C. Binzer, Vertriebs-G.m.b.H., Hatzfeld/Eder, West Germany). With these papers the solvent development times are about 30 h for semi-stench and 18 h for *n*-butanol–propionic acid–water.

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