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THE INFLUENCE OF PEPTIDES ON THE ANALYSIS OF AMINO ACIDS BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The degree of sensitivity and of resolution of amino acids that is possible on cellulose powder makes thin-layer chromatography an ideal system for the analysis of protein hydrolysates. Its application to biological fluids is frequently unsatisfactory, even when salts have been removed, owing to the presence of peptides. The latter may also give rise to ambiguities between amino acids and peptides in the ion-exchange analysis of such fluids. This paper describes how some of these difficulties can be minimised by the use of both techniques concurrently.

INTRODUCTION

The analysis of amino acids is one of the most important fields of biochemistry because of their widespread occurrence in all proteins and biological fluids. Until a few years ago, there was no satisfactory method for the analysis of amino acids on paper, let alone on thin layers. On the qualitative side, there was no satisfactory pair of solvent systems available for separating all the amino acids, including leucine and isoleucine, on the same sheet of paper in less than about 4 days.

Phenolic systems, first used by MARTIN AND SYNGE, are still probably the most effective for the separation of amino acids. However, their corrosive nature, the slow rate of development, the difficulty of removing all the phenol from the paper afterwards, and even the actual destruction of certain amino acids when heated with the phenol, all militate against its use. (Systems based mainly on the lower alcohols, methanol and ethanol, which have been used to overcome these difficulties, have almost invariably resulted in more diffuse spots being formed.) Development with the covalent phase of such diphasic solvent systems as water-saturated phenol or water-saturated butanol have, furthermore, in-built instability and alter in composition with change of temperature. Monophasic systems have always been found, in our experience, to be the most satisfactory in paper chromatography. The use of such solvent systems cannot, of course, be described as simple partition chromatography. The solid phase plays its part and this becomes even more evident when thin layers of cellulose are used.

In order to utilize to the full the property of differing ionic charges between the amino acids as well as that of differing solubility, we have always adhered to the principle of using an acidic solvent in one dimension and a basic solvent in the other — a fundamental principle first exploited most effectively by MARTIN AND

SYNGE, but often forgotten by later workers in their search for better systems.

In the method which we developed[1] in 1967 for the determination on paper of the free amino acids present in gastric juice, the separation was carried out in the first dimension by means of a propan-2-ol–water–ammonia system and in the second dimension with a butanol–acetic acid–water mixture. Later we used the improved solvent systems devised for thin-layer chromatography (TLC)[2,3].

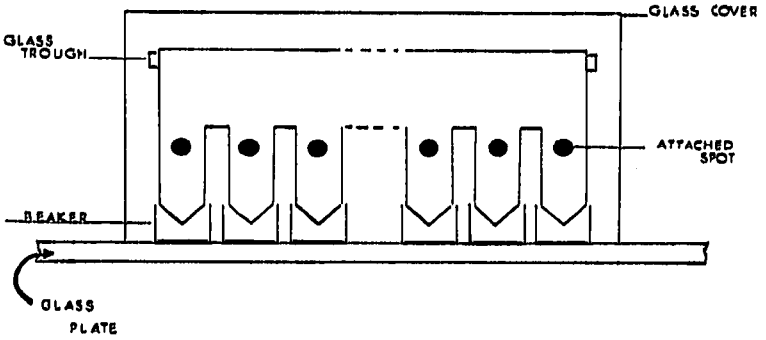

Fig. 1. Elution apparatus, side view.

Also, for quantitative work, there was no satisfactory staining technique that could be applied to the paper. Ninhydrin alone gave very poor results, which at best could be used only as a semi-quantitative estimate.

However, the cadmium acetate–ninhydrin reagent of HEILMAN, BAROLLIER AND WATZKE[4] gave pink-coloured complexes, which could be eluted with methanol in an apparatus such as that shown in Fig. 1.

The great advantage of the paper technique is that a very small amount of protein (about 250 μg) can be analysed to a fairly high degree of reproducibility with the simplest of apparatus — a little glassware and a colorimeter, or even a pair of Nessler tubes, if a spectrophotometer is not available. Hence it is even possible for the analysis of a small peptide to be carried out with reasonable accuracy at the senior level in a secondary school by this technique, which was fully reported earlier[1].

THIN-LAYER CHROMATOGRAPHY OF AMINO ACIDS

The success achieved in the analysis of amino acids on paper encouraged us to see if a similar separation and staining technique could be applied to thin layers of cellulose.

When we commenced this work in about 1964, some attempts had been made to separate amino acids on silica gel but had proved to be unsatisfactory. Work had been carried out with cellulose powder by VON ARX AND NEHER[5], by TEICHERT *et al.*[6], WOLLENWEBER[7] and others, but, despite elaborate multi-development, only the partial separation of some difficult pairs of amino acids had been achieved.

As a starting point, we set ourselves the task of developing a pair of solvent systems that would separate the most difficult pair of amino acids, leucine–isoleucine, on one two-dimensional plate. If this proved to be successful, our further aim was to modify these systems in order to separate all the other amino acids that are likely

to be present in a protein hydrolysate. Both aims were finally achieved[3], and the work resulted in the separation of all the common amino acids, including cystine, cysteine and cysteic acid, on one plate after a total development time of about 5–6 h.

A typical separation is shown in Fig. 2. The method proved to be very useful and because of the greater sensitivity of TLC (10–100 times), we were extremely anxious to make the method quantitative but we realized that, for several reasons, considerable modification would be required. One minor defect in the solvent system was that it relied on a slightly unsaturated atmosphere in the development tank

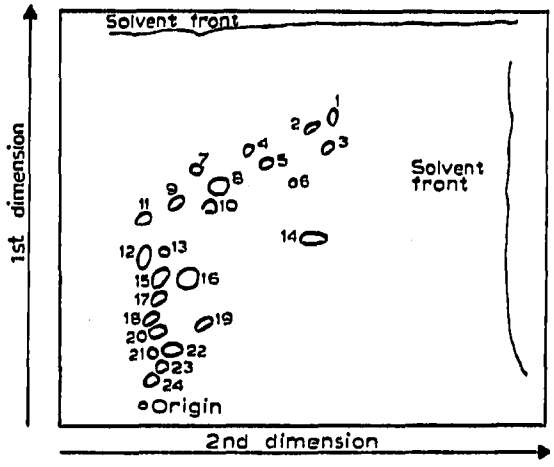

Fig. 2. Amino acid separations on thin layers of cellulose powder[3]. 1 = Leucine; 2 = isoleucine; 3 = phenylalanine; 4 = valine; 5 = methionine; 6 = tryptophan; 7 = 2-amino-*n*-butyric acid; 8 = tyrosine; 9 = α-alanine; 10 = proline; 11 = glutamic acid; 12 = aspartic acid; 13 = hydroxyproline; 14 = threonine; 15 = glycine; 16 = serine; 17 = glutamine; 18 = arginine; 19 = taurine; 20 = lysine; 21 = ornithine; 22 = histidine; 23 = cysteic acid; 24 = cysteine. Solvents: first dimension, propan-2-ol–formic acid–water (40:2:10); second dimension, *tert.*-butanol–methyl ethyl ketone–0.88 NH_3–water (50:30:10:10).

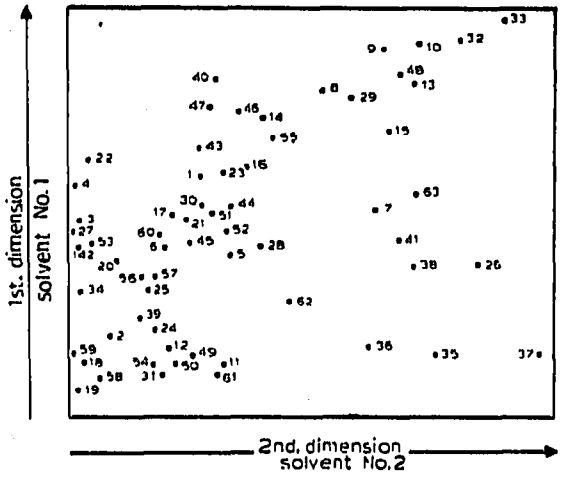

Fig. 3. Map of 63 amino acids and related compounds separated by TLC[3] (for key, see Table I).

TABLE I

R_F × 100 VALUES OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

No.	*Amino acid*	R_F × 100 *value*	
		Solvent No. 1	*Solvent No. 2*
1	Alanine	57	23
2	Arginine	19	6
3	Aspartic acid	48	1
4	Glutamic acid	56	1
5	Serine	39	27
6	Glycine	37	16
7	Threonine	51	61
8	Valine	79	44
9	Isoleucine	90	63
10	Leucine	90	69
11	Histidine	11	26
12	Lysine	16	17
13	Phenylalanine	82	67
14	Tyrosine	72	35
15	Tryptophan	70	54
16	Proline	58	30
17	Hydroxyproline	48	17
18	Cysteine	12	5
19	Cystine	6	3
20	Cysteic acid	53	8
21	β-Alanine	46	19
22	α-Aminoadipic acid	62	3
23	β-Aminoisobutyric acid	60	26
24	Asparagine	21	14
25	Glutamine	31	13
26	Ethanolamine	39	81
27	Phosphoethanolamine	43	0
28	Taurine	41	33
29	Methionine	78	51
30	γ-Amino-n-butyric acid	51	23
31	Hydroxylysine	10	17
32	Norleucine	92	73
33	2-Amino-octanoic acid	93	81
34	Argininosuccinic acid	31	0
35	Cadaverine	14	70
36	Putrescine	16	60
37	Histamine	15	85
38	Kynurenine	38	67
39	Homoarginine	23	12
40	p-Aminohippuric acid	82	25
41	4-Amino-5-imidazole-carboxylic acid	43	63
42	δ-Aminolaevulinic acid	41	2
43	α-Amino-n-butyric acid	65	29
44	Methionine sulphone	50	27
45	Methionine sulphoxide	39	20
46	α-Aminoisobutyric acid	74	30
47	ε-Aminocaproic acid	76	23
48	Ethionine	83	66
49	1-Methylhistidine	14	18
50	3-Methylhistidine	12	14
51	Sarcosine	48	24
52	Homoserine	45	27
53	3,4-DOPA	43	6

(*Continued on p. 185*)

TABLE I (*continued*)

No.	*Amino acid*	$R_F \times 100$ *value*	
		Solvent No. 1	*Solvent No. 2*
54	Ornithine	11	15
55	Pipecolic acid	66	36
56	Citrulline	34	12
57	Penicillamine	34	15
58	Djenkolic acid	9	5
59	2,6-Diaminopimelic acid	16	1
60	Formiminoglycine	45	16
61	2,4-Diaminobutyric acid	10	25
62	Glucosamine	20	38
63	Epinephrine	51	52

to achieve good separation (presumably slight changes towards a less volatile composition improved the later separation of the more covalent amino acids).

This rendered the solvent system slightly vulnerable to the effects of temperature fluctuations.

A more serious drawback was the fact that, following removal of the second solvent, a slight but variable pink background remained on the plate after staining with the quantitative reagent. This was traced to residual ammonia being left on the plate, presumably due to being trapped by trace amounts of strongly adsorbed formic acid. Reversal of the order of developing solvents so that this could not happen, however, resulted in much poorer separation, particularly of the leucines. When inorganic acids such as HCl were used instead of formic acid, this effect did not occur.

Accordingly, a further study was initiated with the express purpose of improving the JONES AND HEATHCOTE[8] system in respect of rendering the second solvent monophasic over a larger temperature range and therefore capable of operating in a closed system (saturated tank). Additions of acetone and methanol, these compounds being completely miscible with water, were useful for this purpose. After many experiments in which one parameter was varied at a time, a qualitative system was finally devised[8] that resolved 63 amino acids or related N-containing metabolites (see Fig. 3 and Table I). Of these 63 compounds, only hydroxylysine and ornithine could not be separately identified by position alone.

The range of compounds that can be identified on one plate has since been extended to 76 by making use of selective staining[9].

QUANTITATIVE ANALYSIS OF AMINO ACIDS

In order to obtain accurate results in the analysis of proteins, it was necessary to obtain the maximum concentration of colour density and to adjust the positions of the more common amino acids so as to allow maximum separation. Further alterations in the composition of the second solvent were made, therefore, in order to produce a good system for quantitative analysis.

The composition of the new solvent (No. 2) was 2-methylbutan-2-ol–butanone–propanone–methanol–water–ammonia (sp. gr. 0.88) (50:20:10:5:15:5).

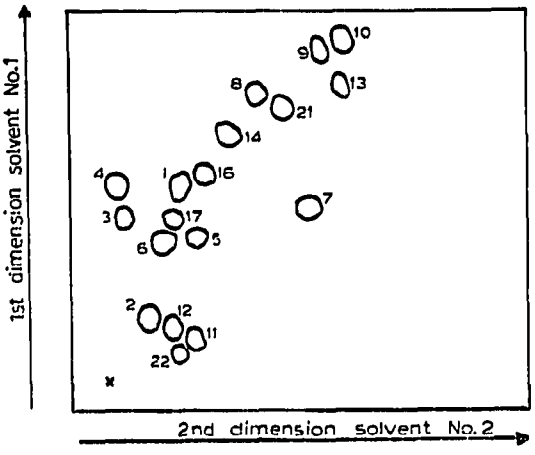

Fig. 4. Separation of amino acids present in a gelatin hydrolysate. Key: 1–20 as in Table I; 21 = methionine; 22 = hydroxylysine.

Fig. 4 and Table II illustrate the results obtained with a gelatin hydrolysate. Consistently good recoveries were obtained[10].

Nowadays there is a tendency to imagine that, provided that the worker has an automated ion-exchange column, the analysis of amino acids is no longer a problem. This may be true of most protein hydrolysates, but in biological fluids many ambiguities arise, and it is always useful to have an alternative, independent method available.

TABLE II

THE AMINO ACID COMPOSITION OF A GELATIN HYDROLYSATE BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

Amino acid	*Amount of amino acid per 100 g of gelatin on a moisture- and ash-free basis (g)*		
	TLC	*Ion-exchange (Technicon)*	*Literature (Ref. 11)*
Alanine	10.6	10.3	9.3
Arginine	8.0	7.6	8.55
Aspartic acid	6.1	6.4	6.7
Glutamic acid	13.0	12.6	11.2
Glycine	28.6	31.1	26.9
Histidine	0.3	0.3	0.73
Hydroxylysine	1.5	1.4	1.2
Hydroxyproline	14.5	14.0	14.5
Isoleucine	1.0	0.8	1.8
Leucine	3.2	3.9	3.4
Lysine	4.4	4.5	4.6
Methionine	0.7	0.8	0.9
Proline	15.3	15.0	14.8
Phenylalanine	2.3	2.0	2.5
Serine	3.2	3.6	3.18
Threonine	1.8	2.2	2.2
Tyrosine	0.2	0.4	1.0
Valine	3.8	3.5	3.3

INFLUENCE OF PEPTIDES ON THE ANALYSIS OF AMINO ACIDS IN BIOLOGICAL FLUIDS

One of the main difficulties in analysing amino acids in biological fluids by paper, and thin-layer, chromatography has been the distortion of the pattern of separation which has rendered quantitative determinations unsatisfactory. This has generally been regarded as being due to the presence of salts, but a more serious interference is usually caused by the presence of peptides in biological fluids. Some of these difficulties, and a method for removing the interference, are described in more detail in the following paper. This has been exemplified by a study on the protein-free filtrate of gastric juice[12].

However, some of the ambiguities in the analysis of biological fluids for amino acids by ion-exchange chromatography may also arise from the presence of unknown (and often unrecognized) peptides in such preparations. In order to obtain some information on this problem, we recently initiated a study of the behaviour of some small (chiefly di-) peptides in both thin-layer and ion-exchange chromatography. A preliminary report has already been published[13] and the results show some very interesting conclusions. Briefly, the main findings are as follows.

Thin-layer chromatography

The R_F values for any series of dipeptides of constant N-terminal residue were influenced by the nature of the C-terminal (amino acid) residue (Fig. 5). For a constant C-terminal residue, the R_F values tended to increase as the N-terminal amino acid increased in molecular size. The effect, which is observed also with simple amino acids, is probably due to the increase in solubility in the mobile organic phase, which is brought about by the increasingly covalent character of the N-terminal amino acid residue.

The N-terminal amino acid appears to exert an appreciable influence on the observed colour of the cadmium–ninhydrin complex formed by a peptide. For example,

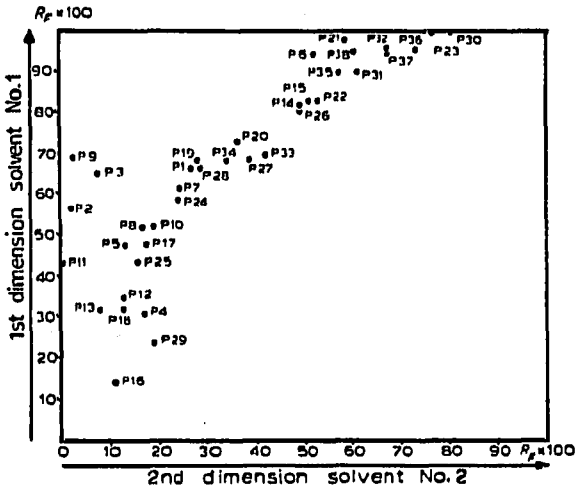

Fig. 5. Map of model peptides after chromatography on thin layers of cellulose (for key, see Table IV).

all the peptides with a glycyl N-terminal residue were found to give a yellow colour similar to proline with the detection reagent. Again, when the positions of the N-terminal and C-terminal residues are reversed in a dipeptide, the observed colour is determined by the nature of the new N-terminal amino acid residue. (The peptides that contain proline seem to be an exception, see Table III.)

TABLE III

INFLUENCE OF N-TERMINAL AMINO ACID RESIDUE ON THE COLOUR OF THE CADMIUM–NINHYDRIN COMPLEX

Peptide	*Colour*	*Peptide*	*Colour*
Gly–Ala (P10)	Yellow	Ala–Gly (P4)	Red
Gly–Val (P20)	Yellow	Val–Gly (P27)	Red
Gly–Leu (P15)	Yellow	Leu–Gly (P22)	Red
Gly–Ser (P18)	Yellow	Ser–Gly (P25)	Orange
Gly–Pro (P17)	Yellow	Pro–Gly (P24)	Mauve

The magnitude of the colour yield also seems to be influenced by the N-terminal residue. In the case of the N-terminal valyl peptides, the colour yields are so low that such peptides would not easily be detected in the routine TLC analysis of biological fluids.

Ion-exchange analysis

Table IV shows the retention factor, R_{AH}, and colour yield for each peptide at the standard (Technicon) wavelengths of 440 and 570 nm. The corresponding values for many common amino acids are included for comparison and a diagram showing the relative positions of some of the eluted peaks is given in Fig. 6.

The order of elution of the dipeptides from the column was almost invariably governed by the C-terminal residue. Thus for the series of dipeptides with N-terminal glycine, the order of elution is the same as that for the C-terminal amino acids aspartic acid, glycine, alanine, valine, tyrosine and lysine. The peptides glycylserine and glycylproline occupy unexpected positions.

All the peptides examined were retained on the column much longer than the amino acid corresponding to the C-terminal amino acid, and increasing retention occurred with increasing complexity of the molecule and especially of the N-terminal residue.

The colour yields of the valyl peptides are very low and column loadings in excess of 0.5 μmole were needed in order to obtain satisfactory peaks. Such peptides would not be readily detected in the routine analysis of a biological fluid. The dipeptides prolylglycine and alanylproline do not have any detectable absorption at wavelengths of 440 and 570 nm and consequently no position of elution can be recorded. In contrast with alanylproline, valylproline has an appreciable colour.

It can be seen from Table IV and also Fig. 6 that many peptides have elution positions that coincide with the more common amino acids and these could readily be mistaken for the latter without the corrective adjunct of TLC examination.

The dominant role of the N-terminal amino acid in determining the colour, nature and intensity of the cadmium–ninhydrin complex on TLC is not so evident

TABLE IV

ELUTION PATTERN OF AMINO ACIDS AND PEPTIDES ON ION-EXCHANGE RESIN (TECHNICON)

Peptide or amino acid		$R_{Ala} \times 100$	*Column loading (μmoles)*	*Peak area*[b]		*Colour yield*		
				440 nm	*570 nm*	*(Area per μmole)*		*Area at 570 nm*
						440 nm	*570 nm*	*Area at 440 nm*
Aspartic acid		0	0.10	1.2	7.4	12.0	74.0	6.2
Threonine		3.4	0.10	1.3	5.8	13.0	58.0	4.5
Serine		4.2	0.10	1.3	6.3	13.0	63.0	4.8
Glutamic acid		10.7	0.10	1.6	8.4	16.0	84.0	5.3
Proline		14.1	0.10	1.5	0.3	15.0	3.0	0.2
Glycine		20.8	0.10	1.4	9.2	14.0	92.0	6.6
Gly–Ser	P18	22.2	0.68	2.0	18.4	2.9	27.1	9.3
Alanine		25.3	0.10	1.3	8.0	13.0	80.0	6.2
Ala–Asp	P2	25.4	0.50	2.5	16.8	5.0	33.6	6.7
Gly–Asp	P11	28.8	0.32	0.3	1.8	5.6	56.0	10.0
Ala–Ser	P8	28.9	0.40	1.3	9.8	3.3	24.9	7.6
Gly–Gly	P12	30.6	0.51	2.6	23.7	5.1	46.5	9.1
Ser–Gly	P25	35.3	0.50	3.2	29.0	6.4	58.0	9.1
Glu–Ala	P9	37.1	0.46	10.1	35.3	22.0	77.0	3.5
Ala–Gly–Gly	P5	37.7	0.50	1.5	9.4	3.0	18.8	6.3
Valine		38.8	0.10	1.4	7.9	14.0	79.0	5.6
Ala–Glu	P3	40.7	0.50	2.6	16.5	5.2	33.0	6.4
Ala–Gly	P4	44.0	0.50	3.7	25.2	7.4	50.4	6.8
Cystine		44.5	0.10	1.6	4.1	16.0	41.0	2.6
Val–Ser	P34	45.5	0.50	0.2	1.2	0.4	2.4	6.0
Methionine		46.7	0.10	1.3	8.4	13.0	84.0	6.5
Ala–Ala	P1	47.9	0.37	2.0	16.5	5.4	44.6	8.3
Gly–Gly–Gly	P13	48.2	0.50	2.6	18.4	5.2	36.8	7.1
Gly–Ala	P10	48.5	0.40	2.3	29.5	5.8	74.0	12.8
Val–Gly	P27	54.1	0.50	0.6	2.7	1.2	5.4	4.5
Isoleucine		54.6	0.10	0.9	7.9	9.0	79.0	8.8
Val–Ala	P26	55.3	0.50	0.2	1.2	0.4	2.4	6.0
Val–Gly–Gly	P28	57.4	0.50	0.1	0.6	0.2	1.2	6.0
Leucine		57.5	0.10	1.3	8.3	13.0	83.0	6.4
Gly–Val	P20	58.9	0.46	3.6	30.5	7.8	66.5	8.5
Leu–Ala	P21	59.2	0.50	1.3	9.6	2.6	19.2	7.4
Gly–Pro	P17	62.3	0.46	4.1	34.5	8.9	75.0	8.4
Pro–Gly[a]	P24	—	2.00	—	—	—	—	—
Ala–Pro[a]	P7	—	2.00	—	—	—	—	—
Tyrosine		63.5	0.10	1.8	8.3	18.0	83.0	4.6
Val–Pro	P33	65.8	0.50	4.0	21.7	8.0	43.4	5.4
Phenylalanine		66.3	0.10	1.3	8.0	13.0	80.0	6.2
Leu–Val	P23	66.5	0.50	2.5	8.0	5.0	16.0	3.2
Val–Val	P38	67.0	0.50	0.3	1.3	0.6	2.6	4.3
Leu–Gly	P22	70.2	0.53	4.3	31.2	8.1	59.0	7.3
Gly–Ile	P14	71.6	0.53	5.1	36.8	9.6	69.5	7.2
Gly–Leu	P15	72.4	0.48	4.4	37.4	9.2	78.0	8.5
Val–Met	P31	72.4	0.50	0.5	2.0	1.0	4.0	4.0
Val–Leu	P30	73.8	1.00	1.2	6.5	1.2	6.5	5.4
Gly–Tyr	P19	84.1	0.50	2.8	24.4	5.6	48.8	8.7
Val–Tyr–Val	P36	84.7	1.00	1.0	6.5	1.0	6.5	6.5
Val–Tyr	P35	86.0	0.50	0.3	1.4	0.6	2.8	4.7
Ala–Phe	P6	87.0	0.50	4.4	31.5	8.8	63.0	7.2
Val–Phe	P32	90.0	0.50	0.4	2.1	0.8	4.2	5.3
Lysine		95.0	0.10	2.1	9.5	21.0	95.0	4.5
Histidine		100.0	0.10	1.6	8.7	16.0	87.0	5.4
Val–His	P29	112.5	0.50	0.4	2.4	0.8	4.8	6.0
Gly–Lys	P16	114.0	0.50	4.1	34.6	8.2	69.2	8.4
Val–Trp	P37	121.0	0.50	0.2	1.1	0.4	2.2	5.5
Arginine		122.0	0.10	1.3	7.7	13.0	77.0	5.9

[a] Not observed at a column loading of 2 μmoles.
[b] "True Standard Area" at 570 nm was 8.05.

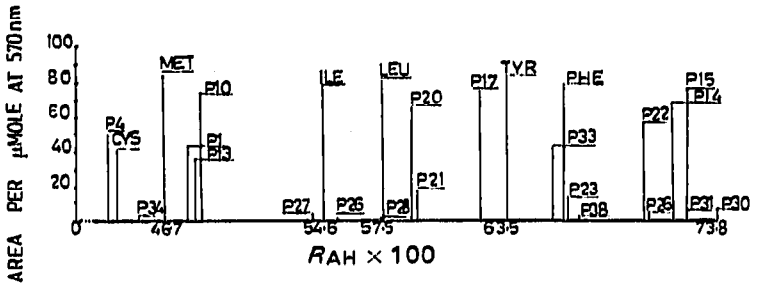

Fig. 6. Ion-exchange chromatography of amino acids and dipeptides (for key, see Table IV).

in ion-exchange chromatography, in which ninhydrin alone is used. It is not known which factors other than the metal ion play a part in the peculiar colour formations observed on TLC but, undoubtedly, the variations in colour given by peptides could be useful guides to sequence structure in simple peptides. R_F values alone are insufficient to characterize peptides, and formulae designed to predict molecular structure by determining small incremental changes in these values are notoriously unreliable. However, R_F values could be valuable guides to structure if taken in conjunction with the colour of complex and the retention factor R_{AH} in a well tried ion-exchange system. The resolution achieved by the amino acid AutoAnalyzer was probably better for the valyl and leucyl peptides than that given by TLC, in which these peptides have high R_F values in both solvent systems. It is concluded that a combination of TLC and ion-exchange analysis is extremely useful for the detection and determination of small unknown peptides.

There is an undoubted advantage in TLC work when only a small amount of protein is available for analysis. A sample of 15 μg of protein hydrolysate should be adequate by densitometry with the Joyce–Loebl Chromoscan, where at least 500 μg would be required in the standard ion-exchange column technique. When an automatic column method is available, the TLC method provides a useful rapid check of the concentration of sample to be loaded on to the column. Numerous samples of biological fluids can be analysed by the TLC method at any given time. Frequently, as with screening tests for pathological urines, only a small percentage of samples is required for quantitative analysis.

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DISCUSSION

HAIS: Dr. HEATHCOTE spoke about a case in which some solutes (peptides) carry along other solutes (free amino acids) to positions different from those in pure solutions. It may be of interest, in this connection, to recall an idea expressed by the originator of chromatography, the centenary of whose birth we have been celebrating recently. In his 1910 monograph, "Chromophylls in the Plant and Animal World", when discussing the resolving power of his method, M. S. TSWETT considered the possibility of the formation of complexes AA, BB and AB from the components (A and B) of a mixture. He admitted that such a phenomenon (similar to the case of eutectics in distillation), if it actually took place, would greatly complicate the separation; various separation methods should therefore be cross-checked*. It is remarkable that TSWETT did not refrain from waging such an important objection against his own cherished method, which, if found to be of general validity and importance, would greatly limit the applicability of the technique. Fortunately, this is not the case, as shown by the present spread of chromatography. Nevertheless, as shown in Dr. HEATHCOTE's paper, there are instances in which mutual interaction of the solutes may cause complications, thus justifying TSWETT's caution.

* M. S. TSWETT; *Khromatograficheskii Adsorbtsionnyi Analiz. Izbrannye Raboty*, Izdat. Akad. Nauk SSSR, Moscow, 1946, p. 143.