THE RELATIVE RATES OF MOVEMENT OF AMINO ACIDS ON ION-EXCHANGE CELLULOSE AND ION-EXCHANGE RESIN LOADED PAPERS

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INTRODUCTION

The pioneerwork of PARTRIDGE *et al.*[1–4], of MOORE AND STEIN[5–8] in the field of amino acid chromatography on ion-exchange materials is well known. During the course of their studies, PARTRIDGE and his colleagues[4] investigated the relationship between ionic and non-ionic properties of an amino acid and its position in the displacement series on strong acid and strong base exchangers. They concluded that the "adsorption affinity" could be regarded as the sum of two factors: the electrostatic attraction of the two charged centres and Van der Waals' forces. The electrostatic attraction is influenced by the valency of the ion, by its hydration and by factors affecting the ionic diameter. Van der Waals' adsorption, on the other hand, will vary with the size and nature of the non-ionic part of the molecule. According to PARTRIDGE, consideration of the displacement series of amino acids on ion-exchange materials confirmed that the main factor determining their sequence was the basic or acidic strength of the amino acid and any departure from such a sequence was regarded as "anomalous".

DAVIES[9], however, pointed out that the operation of a Van der Waals' type of interaction allowed the separation of components of mixtures of amino acids which had too closely similar pK values to allow separation by ionic interaction alone. Other workers have also reported the effect of Van der Waals' interaction on amino acid sequence. CLEAVER AND CASSIDY[10] found that a number of amino acids were sorbed by cation exchange materials at pH values at which only a very small amount of amino acid could be in a cationic form. Amino acids with essentially the same iso-electric point were found to be taken up to different extents. The conclusion reached was that sorption was not of an ionic type. CLEAVER[11] also showed that the sorption of nor-leucine and phenylalanine on cation exchangers followed the order of equivalent weights of the resin. ENGLIS AND FIESS[12] have reported sorption of some amino acids on ion-exchange resins showing the same order as that in which the same amino acids distributed themselves between an aqueous and an organic phase and that the neutral amino acids sorbed to the greatest extent were those which possessed the greatest hydrocarbon portion in the molecule.

It is obviously important, not only in the case of amino acids but also quite generally in ion-exchange involving organic ions, to be able to evaluate the relative effect of ionic and non-ionic interactions under any given set of conditions. Against the background presented above the present work has provided an opportunity for

TABLE

Ion-exchange cellulose papers

Exchanger	*Functional group*	*Matrix of exchanger*	*Ion-exchange capacity of paper* meqiv./sq.cm	*W.R.* value of exchanger*	*Original ionic form of paper*
Cellulose phosphate	Dihydrogen** phosphate	Cellulose	9.1***	1.7–2.2	Ammonium
Carboxymethyl-cellulose	Carboxyl	Cellulose	4.0	2.9–3.2	Hydrogen
Diethylamino-ethyl-cellulose	Tertiary amino	Cellulose	3.3	2.5–3.0	Free base

* W.R. = Water regain, defined as follows. Cation exchangers: the weight of water associated with 1 g dry hydrogen exchanger measured by drying the resin after centrifuging to remove interstitial water. Anion exchangers: the weight of water associated with 1 g dry free base exchanger measured by drying the resin after centrifuging to remove interstitial water.

** Monohydrogen phosphate groups will also be present in cross-linked regions.

*** This figure refers to total strong and weak group capacity.

carrying out such an evaluation by comparing the relative rates of movement of amino acids on exchangers with entirely different matrices, which matrices have offered, in the case of the ion-exchange celluloses, hydrophilic centres and in the case of the ion-exchange resins hydrophobic centres for Van der Waals' interaction. This comparison has only been possible because of the availability of these ion-exchange materials in paper form. As a result of these investigations it has been shown that the effect of the non-ionic interactions has a greater significance than has hitherto been reported.

EXPERIMENTAL

Apparatus

A standard all-glass 20 in. Shandon Chromatotank was used for all chromatograms.

Materials

The ion-exchange papers used and some of their properties are listed in Table I. The resin loaded papers are from a range of Whatman Experimental products.

Amino acid solutions

An 0.2 % solution with respect to each amino acid in 0.1 *N* HCl was applied directly to cation exchange papers and made alkaline with ammonia before application to anion exchange papers.

Ionic conversion of the paper

This was carried out by a descending chromatographic wash with a desired solvent usually for an overnight period. When the paper was required in an initially dry state for chromatography (see below) the final wash was with water.

Techniques of development[13]

(1) *Dry start development.* The amino acid solutions were applied to the dry paper. Usual loadings were between 2 and 10 μg of each amino acid and this was applied

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Ion-exchange resin loaded papers

Resin	*Functional group*	*Matrix of exchanger*	*Ion-exchange capacity of paper mequiv./sq.cm*	*W.R. value of exchanger*	*Original ionic form of paper*
Zeokarb 225	Sulphonic acid	Polystyrene	17.2	0.9–1.1	Sodium
Zeokarb 226	Carboxyl	Polyethylene	40	0.8–1.0	Hydrogen
De Acidite G	Tertiary amino	Polystyrene	16	2.0–2.5	Chloride

as a single spot. With these loadings the size of the spot was not restricted. Repeated application with drying in between was unnecessary. Development was by means of a descending technique. No equilibration in the solvent atmosphere was necessary.

(2) *Wet start development.* Ion-exchange paper which had been previously equilibrated with the developing buffer was removed from the chromatography tank and carefully and evenly blotted. The amino acid solutions were applied as spots to the damp paper which was then returned to the chromatography tank and developed by a descending method for a predetermined time. The solvent front was not visible in this technique.

Locating agents

For cation exchange papers an 0.2% solution of ninhydrin in acetone containing 10% collidine was used. For anion exchange papers an 0.2% solution of ninhydrin in acetone containing 10% glacial acetic acid was used.

DISCUSSION

The influence of ionic and non-ionic interaction between amino acids and exchanger can conveniently be studied by comparing the sequence of amino acids on ion exchangers containing the same or similar functional groups but having these groups attached to different matrices.

Strong cation exchange papers

The performance of different papers[14]. Fig. 1 compares the performance of cellulose phosphate paper and paper loaded with Zeo Karb 225 (X 8), under the same development conditions. The functional groups in each case fall into the strong category and the matrices are in the one case hydrophilic cellulose and in the other case hydrophobic polystyrene. The following effects can be observed:

(1) The bivalent character of the basic amino acids under the conditions used is sufficient to cause them to be more strongly retarded than the neutral and acidic amino acids on each paper. The partial bivalent character of cystine is insufficient, however, to isolate it from the neutral amino acids on the resin loaded paper.

(2) The acidic group of amino acids, that is aspartic and glutamic acid, are retarded to a greater extent than some members of the neutral group on each paper. Under the conditions used for these experiments the acidic amino acids in the external

solution would be almost entirely in their zwitterionic form whereas the neutral amino acids would be approximately 50 % cationic. The "anomalous" positions of aspartic and glutamic acid on exchange materials of this type, *i.e.* strong, high capacity, cross-linked materials might be caused by an ion-exclusion effect which differentiates between the predominantly uncharged amino acids and those carrying a heavier positive charge. This effect, which occurs to a similar extent on each type of paper, has been found to diminish as the zwitterion content of the neutral and acidic groups of amino acids becomes more similar (see p. 209).

(3) The individual members of the neutral and basic groups of amino acids occupy different relative positions on the two papers. In Table II these sequences are compared

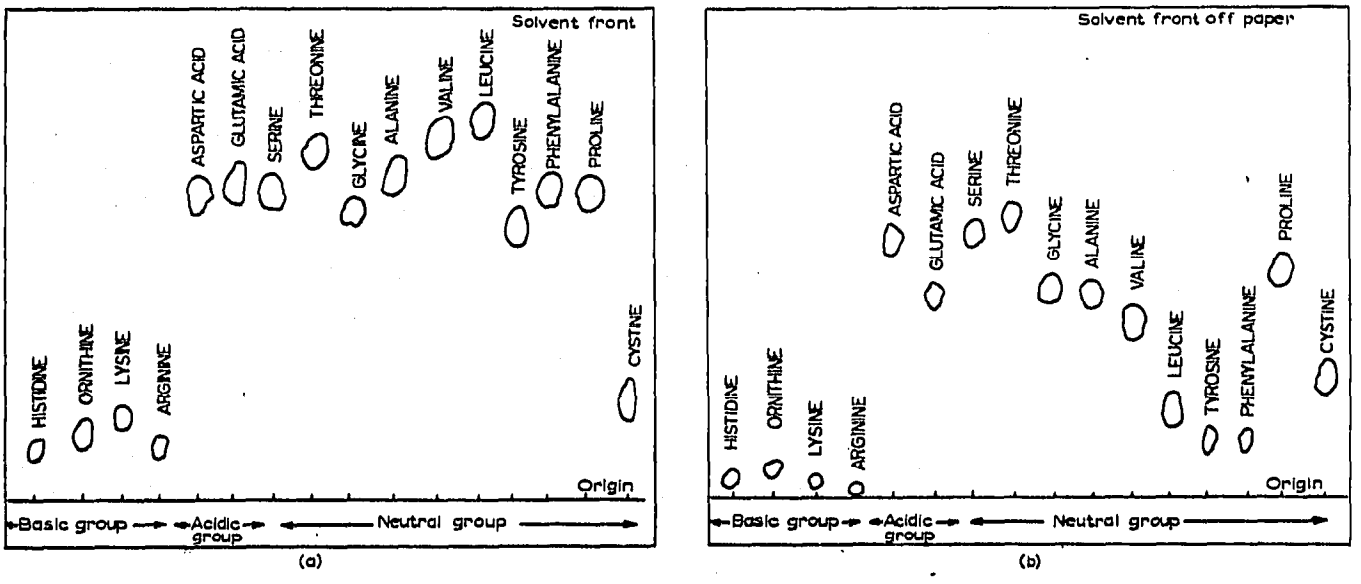

Fig. 1. One-dimensional chromatograms of 16 amino acids on strong cation exchange papers. Paper: (a) cellulose phosphate; (b) Zeo Karb 225 loaded. Developing solvent: 0.2 *M* pH 3.1 sodium buffer. Ionic form of paper: sodium. Development technique: descending dry-start method. Loading: 2 μg of each amino acid.

with the sequences of relevant dissociation constants as compiled by COHN AND EDSALL[15].

Differences in the functional group structure and in the ion-exchange capacity and degrees of cross-linking of the exchangers will contribute to different selectivities on the two types of paper. The nature of the sequence of amino acids illustrated in Fig. 1, however, indicates that within the same group of amino acids differences in sequence are predominantly due to the different exchanger matrices. Thus, the aliphatic side chain amino acids, glycine, alanine, valine, and leucine are arranged in reversed sequence on the two chromatograms. The sequence in each case is consistent with the different degrees of non-ionic interaction between amino acid side chain and exchanger matrix. A similar effect has also caused the neutral amino acids serine and alanine to occupy the reverse positions on the two papers. In this case the reversal is consistent with the replacement of a hydrogen atom in the alanine side chain by a hydroxyl group in the serine side chain. Similarly, within the basic group of amino acids the longer aliphatic side chain of lysine has caused it to be more strongly retarded

than ornithine on the resin loaded paper and the reverse effect to occur on the cellulose exchanger.

The effect of development conditions. The conditions used for the chromatograms shown in Fig. 1 have been close to those required for optimum resolution. The effect of changing the external conditions on the relative influence of the ionic and non-ionic interactions between amino acid and ion exchanger are illustrated in Fig. 2. The effect

TABLE II

Sequence of increasing affinity for exchanger				*Sequence of increasing pK_1 (COOH) values*	
Cellulose phosphate paper		*Zeo Karb 225 loaded paper*			
Leu		Thr			Asp 1.88
Val		Ser			Glu 2.19
Thr					
Ala			Asp	Phe 1.83	
		Pro		Pro 1.99	
	Glu	Gly		Tyr 2.20	
Phe			Glu	Ser 2.21	
Pro				Cys 2.26 (pK_2)	
				Val 2.32	
		Ala		Ala 2.34	
Ser		Val		Gly 2.34	
	Asp	Cys		Leu 2.36	
		Leu			
				Thr —	
Gly					
Tyr		Tyr			
Cys		Phe			
	Lys		Orn		His 1.77
	Orn		His		Arg 2.01
	Arg		Lys		Lys 2.18
	His		Arg		Orn —

of increasing the pH of development from 3 to 4 using the same sodium ion concentration has been to reduce the resolution significantly. The reduction in cationic form of the amino acids which would follow this increase in pH would be expected to cause decreased resolution for the same distance of solvent travel. However, the loss of resolution indicated in Fig. 2 is larger than could be accounted for by this effect alone due to changes in the relative positions of the amino acids. It is apparent that these changes are mainly brought about by a decrease in the degree of non-ionic relative to the ionic interaction between the amino acids and the exchanger at the higher pH.

At pH 4 the acidic amino acids are retarded to a lesser extent relative to the neutral group than they are at pH 3. This change coincides with the zwitterion contents of the two groups in the external solution becoming more similar with the increase in pH (see p. 208).

An extreme example of the influence of external conditions is illustrated in Fig. 3, which shows the effect of increasing the pH of the developing buffer from 3 to 9 on the sequence of the basic amino acids on the two types of paper. In Table III these sequences are compared with those of relevant pK values of the amino acids. It is apparent that as the cationic character of the amino acid decreases so does the

relative degree of non-ionic interaction with the exchanger and at pH 9 the sequence of amino acids is that predicted by the dissociation constants of the relevant groups.

It may be concluded that in the case of strong cation exchangers the non-ionic interaction effect between amino acids and exchanger has little influence on group

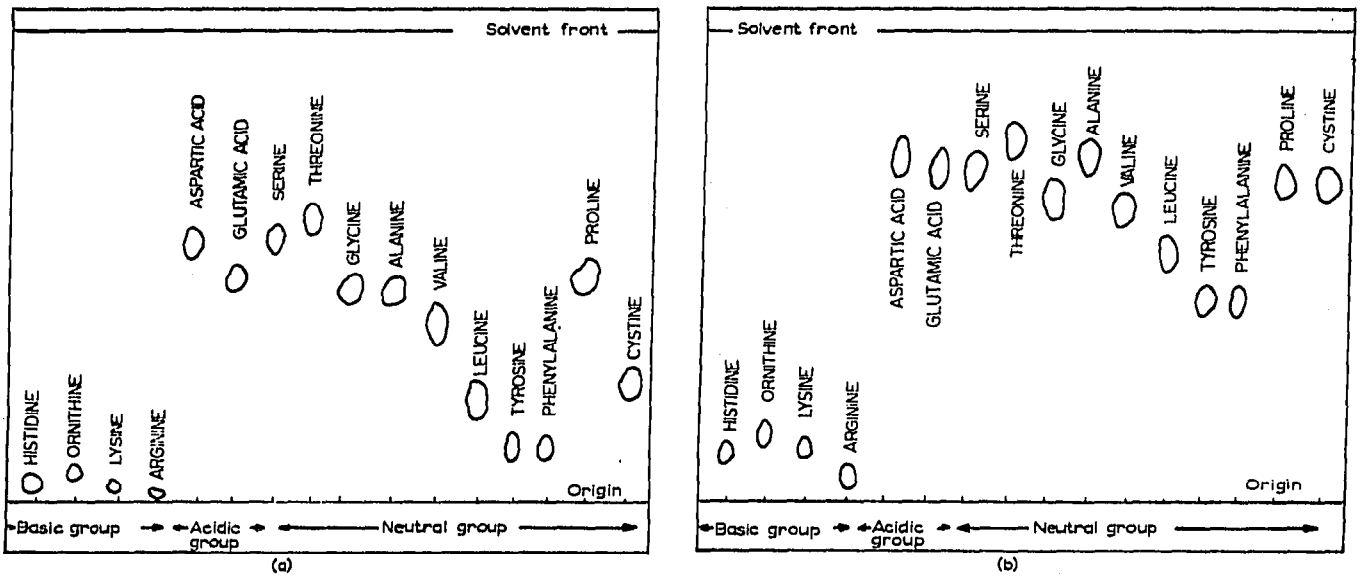

Fig. 2. One-dimensional chromatograms of 16 amino acids on Zeo Karb 225 loaded paper. Developing solvent: (a) 0.2 *M* pH 3.1 sodium buffer; (b) 0.2 *M* pH 4.1 sodium buffer. Ionic form of paper: sodium. Development technique: descending dry-start. Loading: 2 μg of each amino acid.

TABLE III

Sequence of increasing affinity for the exchanger				*Sequence of increasing pK_1, pK_2 and pK_3 values*	
Cellulose phosphate paper		*Zeo Karb 225 loaded paper*			
In 0.2 M pH 3.1	*In 0.2 M pH 9.2*	*In 0.2 M pH 3.1*	*In 0.2 M pH 9.2*	pK_1	pK_2 and pK_3
Lys	His	Orn	His	His 1.77	His 6.00 and 9.17
Orn	Lys	Lys	Lys	Arg 2.01	Lys 8.98 and 10.53
Arg	Orn	His	Orn	Lys 2.18	Arg 9.00 and 12.48
His	Arg	Arg	Arg	Orn —	Orn —

separation which is controlled mainly by ionic interactions. On the other hand, within the individual groups the degree of resolution is very dependent upon non-ionic interaction. The non-ionic interaction is largely responsible for the high degree of resolution within the neutral group of amino acids on the sulphonated polystyrene resin loaded paper and the corresponding lower degree of resolution within this group on the cellulose phosphate paper under the same conditions. However, the relative role of ionic and non-ionic interactions is variable and changes with external conditions.

Highest resolution on both types of paper is consistent with conditions under which the influence of non-ionic interactions is dominant. Such conditions require a high equilibrium concentration of amino acid cation within the exchanger.

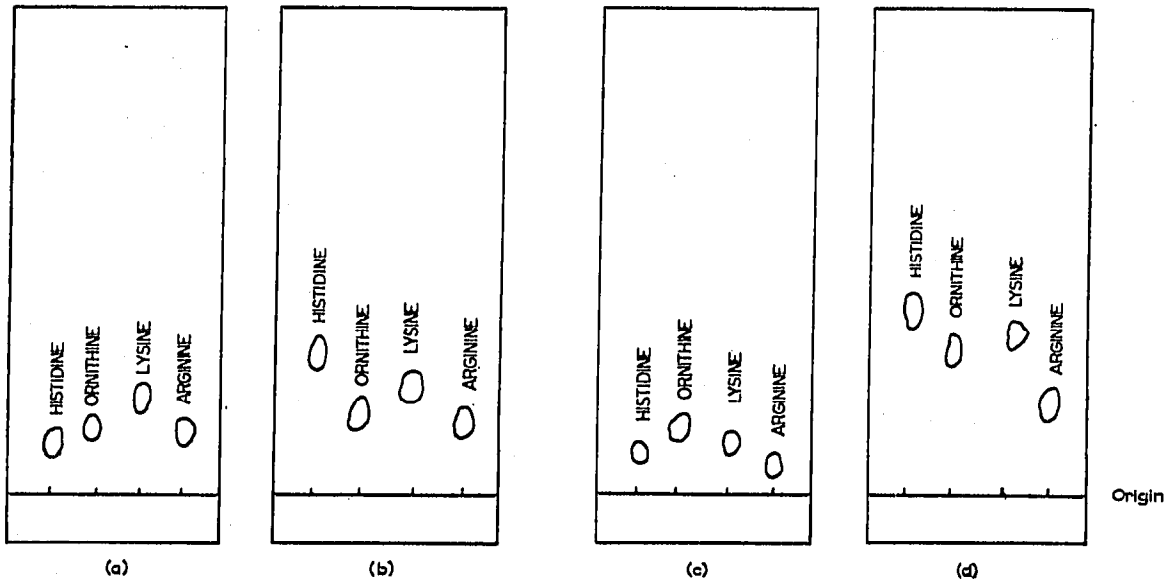

Fig. 3. One-dimensional chromatograms of the basic amino acids on strong cation exchange papers. Paper: (a) and (b) cellulose phosphate; (c) and (d) Zeo Karb 225 loaded. Developing solvent: (a) and (c) 0.2 *M* pH 3.1 sodium buffer; (b) and (d) 0.2 *M* pH 9.2 sodium buffer. Ionic form of paper: sodium. Development technique: descending dry-start. Loading: 2 μg of each amino acid.

Weak cation exchange papers

The performance of different papers. The performances of weak cation exchange papers in which the same functional group, in this case carboxyl, is attached to a cellulose matrix (carboxymethylcellulose, CMC) and a polyethylene matrix (Zeo Karb 226 resin loaded paper) are illustrated in Fig. 4. In Table IV the sequences within selected groups of amino acids on each paper are compared.

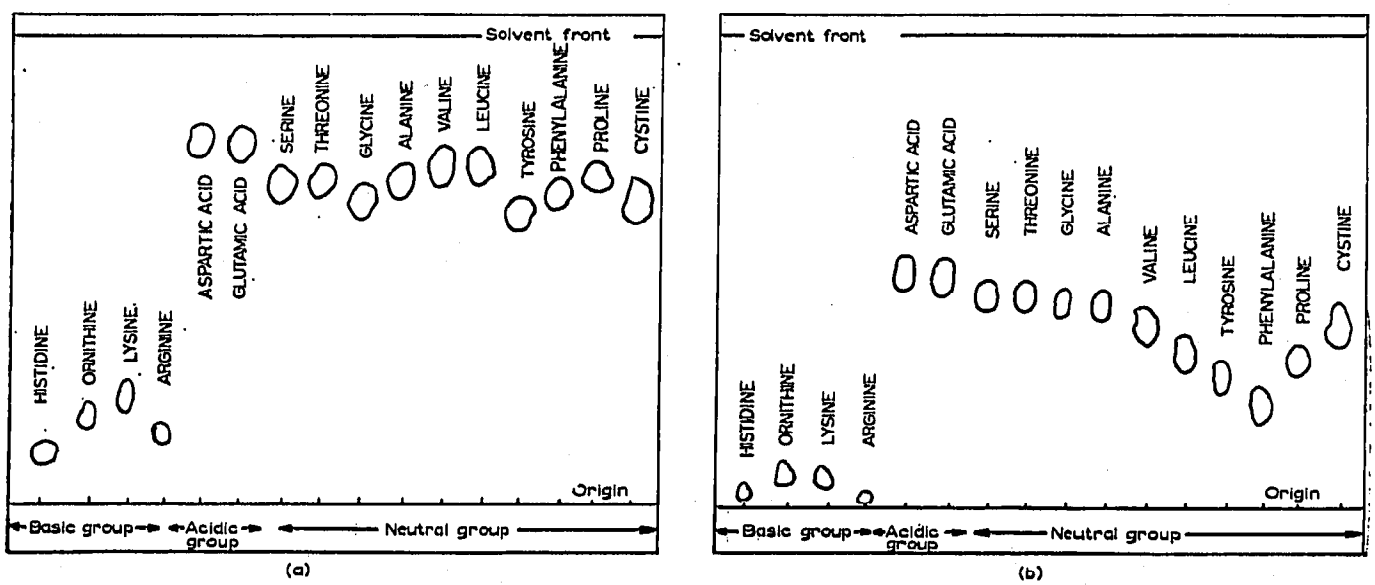

Fig. 4. One-dimensional chromatograms of 16 amino acids on weak cation exchange papers. Paper: (a) carboxymethylcellulose; (b) Zeo Karb 226 loaded. Developing solvent: 0.01 *M* pH 4.5 sodium buffer. Ionic form of paper: buffer equilibrated. Development technique: descending wet-start. Loading: 2 μg of each amino acid.

The group separations on both weak cation papers are as predicted by their ionising characteristics. The complete (on CMC paper) and partial (on Zeo Karb 226 paper) separation of the acidic group is in accordance with the absence of any significant ion-exclusion effect (see p. 208) on weak exchangers under the conditions used. This would particularly apply to CMC paper which, in addition to being a weak exchanger, is of relatively low capacity and is uncross-linked[16].

TABLE IV

Sequence of increasing affinity for exchanger in 0.01 M pH 4.5 buffer						*Sequence of increasing pK_1 values*		
Carboxymethylcellulose			*Zeo Karb 226 loaded paper*					
Aliphatic side chain group	*Aromatic side chain group*	*Basic group*	*Aliphatic side chain group*	*Aromatic side chain group*	*Basic group*	*Aliphatic side chain group*	*Aromatic side chain group*	*Basic group*
Leu	Phe	Lys	Gly, Ala	Tyr	Orn	Val 2.32	Phe 1.83	His 1.77
Val	Tyr	Orn		Phe	Lys	Gly 2.34	Tyr 2.20	Arg 2.01
Ala		Arg			His	Ala 2.34		Lys 2.18
Gly		His	Val		Arg	Leu 2.36		Orn —
			Leu					

The different relative rates of movement of amino acids within the separate groups shown in Figs. 4a and b are of such a nature that they may again be accounted for largely by the different types of non-ionic interaction between amino acid and exchanger which are involved, with the contribution of the different capacities and degrees of swelling of the two exchangers being small in comparison.

The effect of development conditions. The choice of development conditions suitable

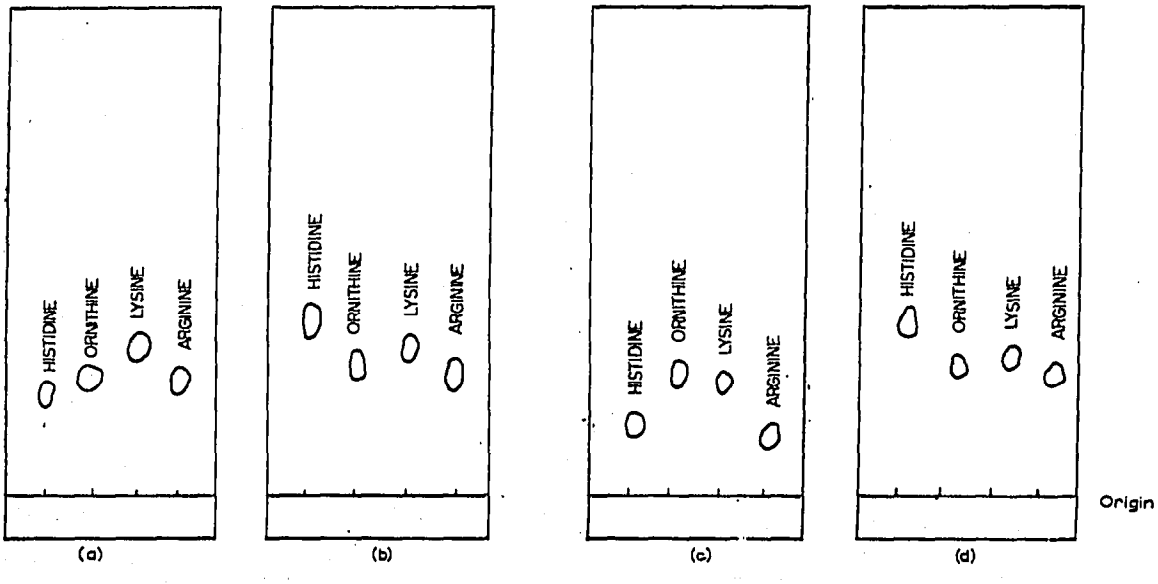

Fig. 5. One-dimensional chromatograms of the basic amino acids on weak cation exchange papers. Paper: (a) and (b) carboxymethylcellulose; (c) and (d) Zeo Karb 226 loaded. Developing solvent: (a) and (c) 0.01 *M* pH 4.5 sodium buffer; (b) and (d) 0.01 *M* pH 9.0 sodium buffer. Ionic form of paper: buffer equilibrated. Development technique: descending wet-start. Loading: 2 μg of each amino acid.

for investigating a full range of amino acids is restricted by the ionising characteristics of the exchangers themselves.

Fig. 5 shows the effect on the sequence of the basic amino acids alone of changing the pH of development from 4 to 9. The sequences are compared with those of relevant pK values in Table V.

The relative rates of movement of these amino acids shown in Fig. 5 are closely similar to those obtained on the strong cation exchange papers under comparable conditions. They illustrate further how the non-ionic interactions between amino

TABLE V

Sequence of increasing affinity for the exchanger				*Sequence of increasing pK_1, pK_2 and pK_3 values*	
Carboxymethylcellulose paper		*Zeo Karb 226 loaded paper*			
In 0.01 M pH 4.5	*In 0.01 M pH 9.0*	*In 0.01 M pH 4.5*	*In 0.01 M pH 9.0*	pK_1	pK_2 and pK_3
Lys	His	Orn	His	His 1.77	His 6.00 and 9.17
Orn	Lys	Lys	Lys	Arg 2.01	Lys 8.98 and 10.53
Arg	Orn	His	Orn	Lys 2.18	Arg 9.00 and 12.48
His	Arg	Arg	Arg	Orn —	Orn —

acid and exchanger dominate the sequence of basic amino acids under acid development conditions when the equilibrium concentration of amino acid cation within the exchanger is high and how ionic interactions dominate the sequence under alkaline development conditions when the equilibrium concentration of amino acid cation within the exchanger is relatively low.

Anion exchange papers

The absence, at the time this investigation was carried out, of a strong anion exchange cellulose paper was a hindrance to a full comparative study of the effect of hydrophilic and hydrophobic exchanger matrices on the performance of anion exchange materials. It was also found that the anion exchange resins of both weak and strong types when incorporated into paper were not particularly suitable for amino acid chromatography. This was attributed mainly to the very slow kinetics which characterised even the most highly porous resins of a quaternary ammonium type and also to the heterogeneity of degree of cross-linking which caused significant variation in performance even among different batches of the same resin. These characteristics persisted despite many attempts to overcome them by varying the physical characteristics of the resin. It has not been possible to date, therefore, to undertake a systematic investigation of the effect of external variables on the performance of strong anion resin loaded papers. A comparative study of the performance of a weak anion resin loaded paper and a weak anion cellulose paper was, however, carried out.

The performance of different papers. Fig. 6 compares the performances of two weak anion exchange papers in which the same functional group, in this case tertiary amino, was attached to a cellulose matrix (diethylaminoethyl (DEAE)-cellulose) and a polystyrene matrix (De Acidite G resin loaded paper).

The group separations on each paper are similar. The acidic group is most strongly retarded because of its partial bivalent character under the conditions used.

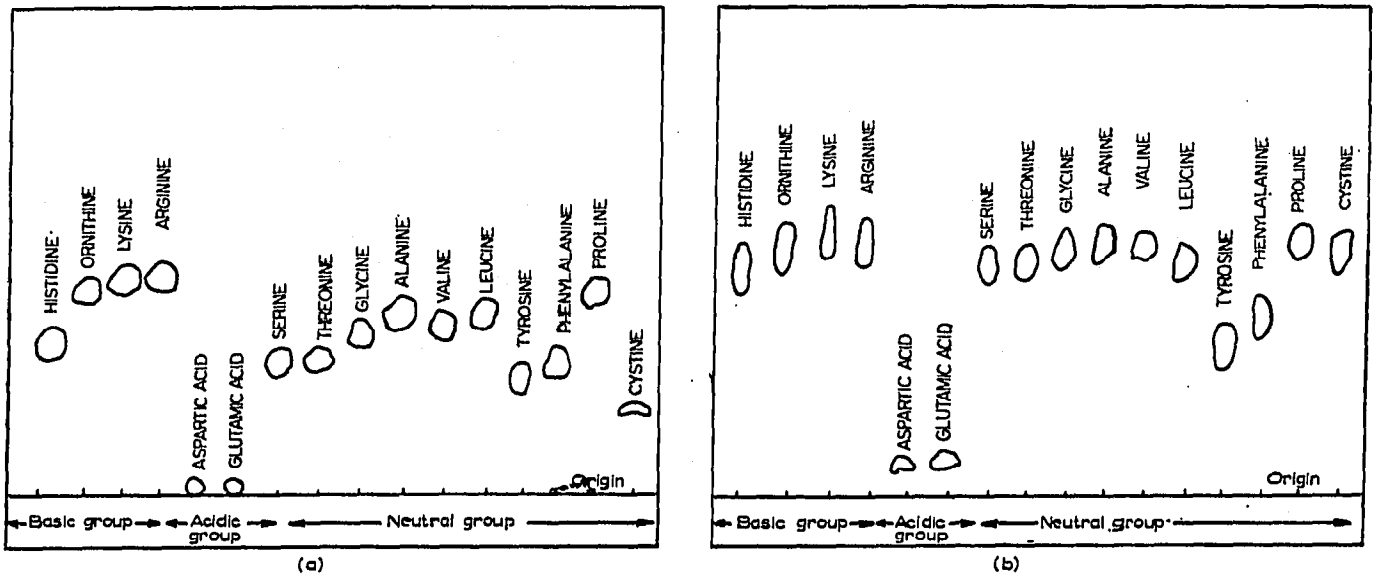

Fig. 6. One-dimensional chromatograms of 16 amino acids on weak anion exchange papers. Paper: (a) DEAE; (b) De Acidite G loaded. Developing solvent: 0.001 *M* sodium chloride. Ionic form of paper: solvent equilibrated. Development technique: descending wet-start. Loading: 2 μg of each amino acid.

The basic group with the exception of histidine, travels further than the neutral group on both papers.

The pH of the developing solvent is close to the iso-electric point of histidine and preferential retardation of this amino acid by means of an ion-exclusion effect would explain its position on the chromatogram on DEAE paper. However, this explanation

TABLE VI

Sequence of increasing affinity for exchanger in 0.001 M NaCl		*Sequence of decreasing pK_a values*
DEAE paper	*De Acidite G loaded paper*	
Arg	Lys	Arg 9.04
	Arg	Lys 8.95
Pro	Orn	His 6.00
		Orn —
Lys	Pro	
Orn	Ala	Pro 10.60
	Val, Cys	Ala 9.87
Ala		Val 9.62
Leu		Leu 9.60
Val	Gly	Gly 9.60
Gly	Leu	Phe 9.24
	Thr	Ser 9.15
His	Ser	Tyr 9.11
	His	Cys 7.85 (pK_3)
Ser, Thr		Thr —
	Phe	
	Tyr	Glu 4.25
Phe		Asp 3.86
Tyr	Glu	
Cys	Asp	
Asp		
Glu		

might be doubted because the low capacity and uncross-linked nature of DEAE cellulose are not compatible with effective ion exclusion[16] and also because a similar effect is not observed on the paper loaded with De Acidite G which has a higher capacity and is cross-linked.

Within the separate groups the differences in relative rates of movement of amino acids shown in Figs. 6a and b are not as striking as in the case of the cation exchange papers but these differences may again be attributed to non-ionic interaction effects rather than to the differing capacities and cross-linking which characterise the two types of exchanger. Thus, the relative rates of movement of the aliphatic side chain amino acids glycine, alanine, valine and leucine on each type of paper differ from those anticipated from pK_2 values in a manner suggesting dependence upon the non-ionic interactions between amino acid and the exchanger. These sequences are compared in Table VI. Similar considerations apply to the relative movement of serine and alanine on the two papers. In the latter instance there is no change in sequence but the degree of separation is higher on the cellulose exchanger in spite of the fact that the DEAE cellulose is uncross-linked and of relatively low capacity compared to the De Acidite G resin.

The rate of movement of cystine on DEAE paper is more consistent with the partially bivalent form of this amino acid which exists at higher pH's.

Reversal of sequences are less prominent in comparing the anion papers than was the case with the cation papers because the differences between the individual pK_2 values are larger than those between the individual pK_1 values (see Tables II and VI). This gives the ionic interaction a stronger control over actual sequences on an anion exchanger compared to a cation exchanger.

The effect of development conditions. The ionising characteristics of the weak

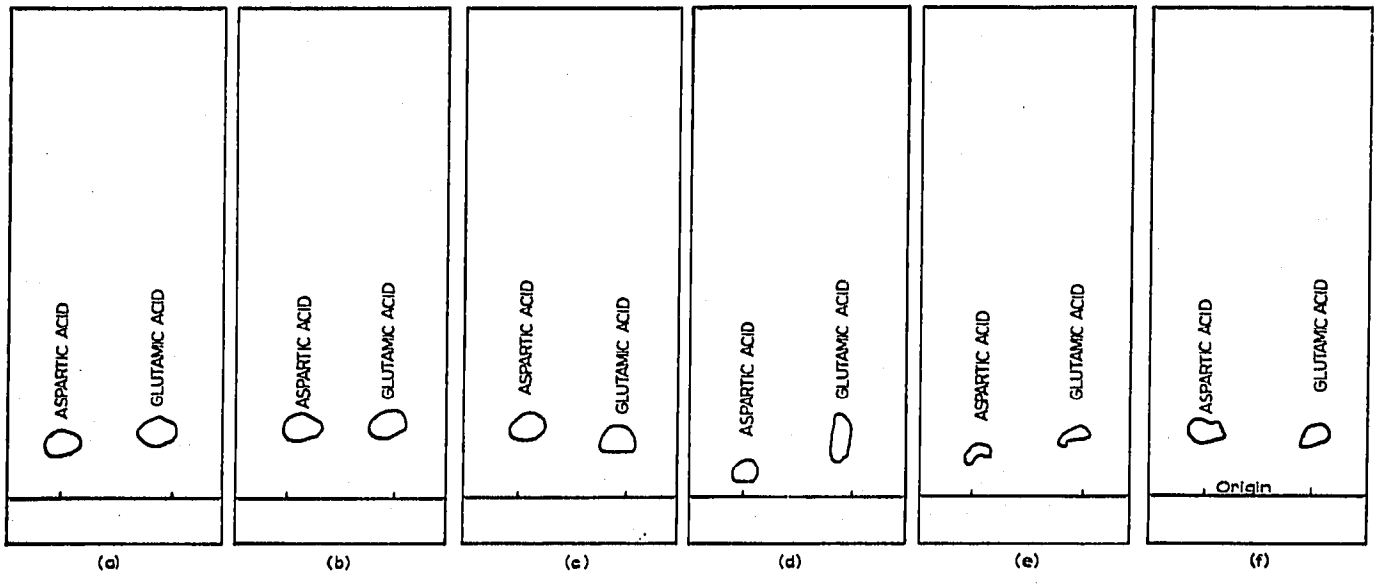

Fig. 7. One-dimensional chromatograms of the acidic amino acids on weak anion exchange papers. Paper: (a), (b) and (c) DEAE; (d), (e) and (f) De Acidite G loaded. Developing solvent: (a) and (d) 0.01 *M* pH 4.5 buffer; (b) and (e) 0.01 *M* NaCl; (c) and (f) 0.01 *M* pH 9.0 buffer. Ionic form of paper: buffer equilibrated. Development technique: descending wet-start. Loading: 2 μg of each amino acid.

anion exchangers prohibit a useful investigation of the relative rates of movement of the full range of amino acids under different development conditions. Fig. 7, however, shows the effect of changing pH of the developing buffer on the relative rates of movement of two acidic amino acids. Under the conditions chosen the sequence of aspartic and glutamic acid on both papers is that predicted by their dissociation constants (see Table VII). Non-ionic interactions between amino acid and exchanger, therefore, play a secondary role in determining the relative rate of movement under these conditions.

It should be emphasised that the chromatograms illustrated in Fig. 6 do not represent optimum performance of the anion exchange papers. The conditions used were dictated by the weak anion resin loaded paper on which the flexibility of per-

TABLE VII

SEQUENCE OF INCREASING AFFINITY FOR THE EXCHANGER

DEAE paper			*De Acidite G loaded paper*			*Sequence of decreasing pK_1, pK_2 and pK_3 values*	
In 0.01 M pH 4.5 buffer	*In 0.01 M NaCl*	*In 0.01 M pH 9.0 buffer*	*In 0.01 M pH 4.5 buffer*	*In 0.01 M NaCl*	*In 0.01 M pH 9.0 buffer*	*pK_1 and pK_2*	*pK_3*
Glu	Glu	Asp	Glu	Glu	Asp	Glu 2.19 and 4.25	Asp 9.82
Asp	Asp	Glu	Asp	Asp	Glu	Asp 1.88 and 3.86	Glu 9.47

formance was much lower than on DEAE paper. On the latter material higher resolution of amino acids than that shown in Fig. 6 has been obtained using dry-start methods of development[17].

The above results have shown that for any particular species of organic ion there exists a combination of functional group type and matrix structure in an ion-exchange material which results in an optimum separation of the individual members of the species. The results with amino acids is one particular instance of this general phenomenon and these have shown that the highest resolution is obtainable on a strong cation exchange resin in comparison with a strong cation exchange cellulose and on a weak anion exchange cellulose in comparison with a weak anion exchange resin. Selectivity may be further varied by changing the capacity and cross-linking of an exchanger and by changing the development conditions. The most careful choice of such variables, however, cannot overcome incompatibility of ionic and non-ionic structures of the exchanger and of the ions being separated.

SUMMARY

The relative rates of movement of amino acids are compared on ion-exchange cellulose and ion-exchange resin loaded papers under comparable conditions. The effect of changing conditions of development on the sequence of amino acids is also described.

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