CIRCULAR PAPER CHROMATOGRAPHY

Part III. R_f Values of Amino Acids and Peptides

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Summary

1. The R_f values on circular filter-paper chromatograms of a number of amino acids in various solvents have been determined. The following solvent mixtures have been studied: *n*-Butanol-acetic acid-water, phenol water, mesityl oxide-formic acid and aqueous acetone.

2. The influence of concentration of amino acids, the distance of the solvent boundary and the distance of the initial spot from the centre of the filter-paper, and temperature on the R_f values have been studied.

3. The R_f values of some of the peptides in *n*-butanol-water solvent mixture are given.

4. The influence of various factors involved in the movement of amino acids on circular paper chromatograms and the mechanism of chromatographic separation of the amino acids in the light of the results obtained in the present investigation are discussed.

In previous reports (Giri, *et al.*, 1952) techniques were described in which circular filter-papers served as the inert support for the separation and identification of amino acids using butanol-acetic acid-water, as solvent. In order to apply the circular paper chromatographic technique to its fullest extent to the amino acid analysis of biological materials, it is desirable to elucidate the various factors which influence the movement of amino acids on circular filter-paper.

The results obtained on the R_f values of amino acids by downward or upward movement of the solvents using filter-paper strips by previous investigators may not apply in detail to the movement of amino acids in circular paper chromatography. The movement of amino acids in the latter case is directed both in the direction of flow of the solvent towards the edge of the paper and also in the direction orthogonal to the direction of flow of the solvent and hence the movement of amino acids may be considered a two-dimensional movement.

The movement of the amino acids on paper is always expressed in terms of their R_f value which is a measure of the rate of movement of the amino

acid relative to the solvent front. It is determined by the ratio of the distance of the movement of the amino acid to the distance of the movement of the solvent. The R_f value is a constant for a particular amino acid, with a particular developing solvent as the mobile phase, provided all other factors particularly the type of paper, temperature, pH of the sample, the mobile phase, the distance moved by the developing solvent front and the extent of equilibration are maintained constant. Many investigators have shown that the R_f values vary considerably, as the movement of the amino acids on paper is influenced by various factors mentioned above, which are very difficult to control in routine work on chromatographic separations. In fact, the irreproducibility of R_f values is one of the limitations of paper chromatography, and it is the result of lack of information on the various factors influencing the R_f values. Bate-Smith and Westall (1950) have discussed the precautions necessary to obtain accurate R_f values. It is, however, not necessary to observe these conditions, in circular paper chromatography for routine chromatographic separation. Nevertheless, for an accurate knowledge of the various factors affecting the R_f values, it is essential to observe these precautions and the present investigation was, therefore, undertaken with a view to study the influence of the nature of the solvent mixture and other factors on the R_f values of the amino acids.

Experimental

The apparatus and general procedure adopted in the present investigation are based on the method described by Giri and Rao (1952). Manipulative details of the technique were described adequately in previous publications and hence need not be detailed here.

Determination of R_f Values

The amino acid solutions were prepared in concentrations of 0·1 per cent. aqueous solutions. About 10 μ l. of the amino acid solution containing 10 μ g. of the acid were applied as a circular spot at the centre of Whatman No. 1 circular filter-paper by means of a small micro pipette. The spot was allowed to dry at room temperature. After inserting the paper ' wick ' at the centre, the paper was irrigated with the solvent. Fresh solvent mixtures were always used for running the chromatogram as the composition of the solvent mixture changed considerably after each development. The irrigation was continued until the solvent boundary had travelled a distance of 6 cm. from the centre. Depending on the solvent and the distance between the surface of the paper and the solvent level, the time required for complete development was about 2 hours. The paper was then removed, the solvent boundary was marked immediately with a pencil, dried at room

temperature and sprayed with 0·1 per cent. ninhydrin reagent in acetone and dried at 35–40° C. for 30 minutes to develop the colours. Throughout this paper, the classical R_f values, defined as the ratio of the distance moved by a given band of the amino acid to the distance moved by the solvent front, is used. The R_f values are the average of three to four determinations, the distances being measured from different points on the circumference of the solvent boundary. The R_f values presented in the table are subjected to $\pm$ 10 per cent. variation.

Solvents

The various solvent mixtures that were used in the present investigation are described below. It is always desirable to use freshly prepared solvent mixtures as esterification is likely to occur in some solvent mixtures such as butanol-acetic acid mixtures on keeping for some time and also the composition of the solvent mixture is altered after use for development.

Phenol Solvent.—Water saturated phenol solution was prepared by adding 100 c.c. of distilled water to 300 g. of phenol. The mixture was allowed to remain in a separating funnel until the two phases—phenol saturated water layer and water saturated phenol layer separated. The water saturated phenol layer was removed and used for the experiments. Phenol was always distilled before use.

n-Butanol-Acetic Acid-Water Solvent.—40 c.c. *n*-butanol, 10 c.c. glacial acetic acid and 50 c.c. water. Before use this mixture was shaken thoroughly and allowed to stand for some time. The lower layer was discarded. It is desirable to redistil butanol, as slight variations in the rate of movement of the amino acids occur when impure solvents are used without distillation.

Mesityl Oxide-Formic Acid-Water.—This solvent was prepared according to the method described by Bryant and Overell (1951) by shaking one volume of mesityl oxide with one volume of formic acid (85 per cent.) and two volumes of water. The solvent mixture was always prepared fresh immediately before use, as mesityl oxide in presence of the acid gradually undergoes oxidation and polymerisation on keeping with the formation of coloured substances. Mesityl oxide was redistilled and fractionated before preparing the solvent mixture.

Acetone

Acetone was distilled and used after mixing with water in various proportions.

R_f Values for Amino Acids in the Various Solvents Employed

The R_f values of various amino acids in the solvents tested are presented in Table I. They represent the average of values of at least three

TABLE I

Rf Values of Amino Acids

No.	Amino acids	N-Butanol-acetic acid-water		Phenol		Mesityl oxide	
		Circular paper chromatography	Paper strip chromatography (Block, *et al.*, 1952)	Circular paper chromatography	Paper strip chromatography (Rockland, *et al.*, 1951)	Circular paper chromatography	Paper strip chromatography (Bryant and Overell, 1951)
1	Alanine ..	0·45	0·39	0·72	0·66	0·63	0·35
2	Arginine ..	0·32	0·19	0·63	0·58	0·42	0·19
3	Asparagine ..	0·32	..	0·59	..	..	..
4	Aspartic acid ..	0·37	0·33	0·45	0·25	0·50	0·17
5	Cystine ..	0·20	0·17	..	..	0·34	0·06
6	Glutamic acid	0·44	0·37	0·55	0·40	0·53	0·20
7	Glycine ..	0·37	0·33	0·57	0·48	0·57	0·22
8	Histidine ..	0·28	0·19	0·73	0·53	0·33	0·06
9	Iso-leucine ..	0·75	0·68	0·90	0·88	0·87	0·70
10	Leucine ..	0·78	0·72	0·89	0·91	0·88	0·71
11	Nor-leucine ..	0·80	..	0·92	..	0·88	..
12	Lysine ..	0·28	0·18	0·53	0·42	0·58	0·23
13	Methionine ..	0·75	0·57	0·90	0·83	0·81	0·61
14	Ornithine ..	0·25	..	0·43	..	0·40	0·08
15	Phenyl alanine	0·75	0·66	0·91	0·90	0·90	0·72
16	Proline ..	0·45	..	0·91	0·88	0·66	0·39
17	Serine ..	0·31	0·31	0·56	0·42	0·56	0·20
18	Threonine ..	0·40	0·36	0·67	0·58	0·64	0·26
19	Tryptophan ..	0·69	0·61	..	0·88	0·93	0·81
20	Tyrosine ..	0·57	0·53	0·75	0·68	0·83	0·63
21	Valine ..	0·72	0·56	0·86	0·82	0·77	0·58

determinations. For convenience in evaluating the values obtained by the circular paper chromatographic technique the results are compared with those obtained by other investigators using the one-dimensional technique of paper chromatography.

It can be seen from the results that the R_f values of the amino acids as determined by the circular paper chromatographic technique are higher than those obtained by the widely used unidimensional chromatographic techniques. The values reported in the present investigation using *n*-butanol-acetic acid-water as solvent are not exactly similar to those reported earlier by Giri (1951). The cause for this discrepancy in the results is due to the fact that the influence of solvent distance factor was not realised before and as such the distance travelled by the solvent was not fixed. Further, reproduction of R_f values very accurately is difficult, as the movement of the amino acids is influenced by several variable factors which are difficult to control, particularly when the chromatograms are run on different days for longer or shorter periods of time. The R_f values should, therefore, be considered as mere indication of the relative positions of the acids with respect to each other.

Among the solvent mixtures investigated *n*-butanol-acetic acid-water proved to be the most effective solvent for the separation of amino acids. Distinct and well-defined circular bands were obtained with this solvent mixture. On the other hand, somewhat diffused bands were obtained when phenol and mesityl oxide were used as solvents. From the results of the R_f values given in Table I, it can be seen that useful separation of amino acids can be achieved by the choice of suitable solvents. Further, some of the amino acids which cannot be separated by using a particular solvent mixture, can be separated by other solvent mixtures. This is clearly shown in the case of glutamic acid and threonine. These amino acids overlap each other and appear as one band when butanol-acetic acid-water mixture is used as solvent. These amino acids can, however, be easily separated by running the chromatogram with phenol as solvent.

Water-Miscible Solvents.—The solvents which are commonly used for development of chromatograms are non-polar or slightly polar liquids. Consden, Gordon and Martin (1944) suggested that water-miscible solvents can be used provided the water content of the solvent is not too high. They, however, observed that the amino acid bands were broader than those obtained by using immiscible solvents. This was attributed to the variation in the composition of the phases caused by the presence of the amino acids. Water-miscible solvents have been used by Arden, *et al.* (1948) for the sepa-

ration of inorganic ions. Hanes and Isherwood (1949) have also used water-miscible solvents for the separation of phosphoric esters. They found that increase in the water content of the solvent increased the R_f values but at the same time decreased the differentiation between the individual esters. Similar observations were made by Jermyn and Isherwood (1949) for sugars. Brown (1951) from his investigation on the application of paper chromatography to the separation of non-volatile carboxylic acids, showed that the R_f values were increased with increased water content of the solvent, so that the separation of acids with similar R_f values could be improved by adding water to the solvent. Kennedy and Barker (1951) used aqueous ethyl alcohol-ammonia solution, and aqueous acetone-ammonia for the separation of volatile acids by paper chromatography and found that the R_f values could be altered considerably by varying the percentage of water. In general, the addition of a larger proportion of water to the mixture caused the organic acids to travel more rapidly on the chromatogram. Bentley and Whitehead (1950) have employed successfully the water-miscible solvents—methanol, ethanol, *n*-propanol, acetone, pyridine, tetrahydrofuran, furfuryl alcohol and tetrahydrofurfuryl alcohol for the separation of amino acids by paper chromatography. Acetone-containing water (40% v/v) was found to be particularly useful, replacing collidine as a second solvent for chromatograms run first with phenol. Rockland, *et al.*, (1951), investigated the influence of water content of eight water-miscible solvents on R_f values of amino acids and found that the R_f values varied with the percentage of water in the solvent mixture. The R_f values of arginine, histidine and lysine were found to decrease linearly with increasing propanol in the solvent mixture, while the values for aspartic acid and glutamic acid were found to increase linearly in the solvent mixtures containing more than 50% propanol. The R_f values of leucine, cystine, isoleucine, valine, phenyl alanine and tryptophan were found to be minimum at about 50% propanol. The values for alanine, glycine, serine and threonine were not altered very much by change in composition of the solvent. Burma and Banerjee (1951) studied the rate of movement of the amino acids with isopropyl alcohol-water mixtures of varying water content and observed that the R_f values increased and the differentiation between the individual amino acids decreased with increase in water content of the solvent. Kowkabany and Cassidy (1952) also observed that with miscible pairs of solvents, all R_f values, in general, increased with increasing water content and reaching 1·0 with pure water, except phenyl alanine and histidine which gave lower R_f values, 0·95 and 0·65 respectively, thereby indicating that adsorption on the filter-paper might be the cause for the lower values obtained.

The R_f values for the amino acids with aqueous acetone of varying amount of water are given in Table II.

TABLE II

Effect of Water Content of Acetone on the R_f Values of Amino Acids at Room Temperature

No.	Amino acids	Acetone : Water						
		30 : 70	40 : 60	50 : 50	60 : 40	70 : 30	80 : 20	90 : 10
1	Alanine ..	·93	·91	·81	·65	·63	·43	..
2	α-Amino-butyric acid ..	·95	·93	·91	·90	·93	·94	..
3	Arginine ..	·58	·50	·45	·41	·28	..	..
4	Asparagine ..	·95	·97	·99	·92	·91	..	..
5	Aspartic acid ..	1·0	1·0	·93	·86	·86	·82	..
6	Cystine ..	·87	·84	·82	·78	·78	·59	..
7	Glutamic acid ..	·68	·73	·70	·65	·67	·41	..
8	Glycine ..	·98	·95	·84	·83	·80	·75	·39
9	Histidine ..	·83	·81	·78	·75	·71	·53	·37
10	Iso-leucine ..	1·0	·97	·94	·92	·92	·90	·87
11	Leucine ..	1·0	·93	·92	·93	·95	·93	·93
12	Lysine ..	·40	·38	·38	·37	·36	·33	..
13	Methionine ..	·85	·83	·96	·78	·76	·62	·47
14	Nor-leucine ..	1·0	·97	·95	·92	·91	·82	·80
15	Ornithine ..	·57	·40	·38	·38	·37	·36	·33
16	Phenyl alanine ..	·88	·88	·90	·99	·90	·87	..
17	Proline ..	·93	·91	·91	·91	·90	·82	·40
18	Serine ..	·80	·79	·70	·80	·38	·38	·32
19	Tryptophan ..	·81	·79	·78	·75	·76	·78	·80
20	Tyrosine ..	·90	·82	·82	·80	·77	·75	·33
21	Valine ..	·86	·86	·84	·83	·80	·77	·33

It can be seen from the results in Table II that R_f values vary with the amount of water present in acetone, increasing with increasing water content. When pure acetone was used no movement of the amino acids was observed. Acetone containing up to 70 per cent. water has been used. At this high level of water content the difference in R_f values is very little and the bands are very broad, but at lower water content there is some differentiation of R_f values and the bands are reasonably compact. The optimum water content for sharply defined bands lies within the range 20–40% (v/v). One interesting feature is the marked difference in the R_f values of the basic amino acids (arginine, lycine and ornithine) and other amino acids. The R_f values of these three amino acids are consistently very much lower than

those of the other amino acids. These basic amino acids can, therefore, be separated from all other amino acids by aqueous acetone as solvent.

Influence of Concentration of Amino Acids on R_f Value

Fisher and Parson (1948) observed that the R_f value changed with concentration of amino acid. Kowkabany and Cassidy (1952) have shown that the R_f values were only slightly decreased with the increase in concentration of amino acid. The influence of concentration of amino acids on the R_f values of four amino acids determined by circular paper chromatography was investigated. 10 μ l. of each amino acid of varying concentration were spotted on the circumference of a circle (2·5 cm. diameter) drawn from the centre of the filter-paper and the R_f values were determined after developing the chromatogram. The results are presented in Table III.

Table III

Effect of Concentration on the R_f Values of Amino Acids

(Solvent: Butanol acetic acid water)

Distance of solvent boundary from the initial spot — 8 cm.

No.	Amino acids	·1%	·2%	·3%	·4%	·5%	·6%	2·4%
1	Glycine ..	·41	·40	·40	·41	·40	·40	·41
2	Glutamic acid ..	·46	·45	·45	·46	·45	·46	·46
3	Leucine ..	·78	·79	·78	·80	·79	·78	·79
4	Histidine ..	·34	·35	·35	·33	·34	·35	·34

The results show that increase in the concentration of the amino acids has no effect on R_f values of the amino acids, as the values remain unchanged irrespective of the concentration of the amino acids.

Effect of the Distance Moved by the Solvent on the R_f Value

10 μ l. of each amino acid containing 10 μg. were spotted at the centre of the filter-paper and irrigated with the solvent butanol-acetic acid-water. Several chromatograms were run with varying distances of the solvent boundary and the R_f values were determined after development of the chromatogram. The results are presented in Table IV.

The results show that the R_f values of all amino acids vary with the increase in the distance of the solvent boundary from the centre of the filter-paper. The R_f values increase at first with the increase in the distance of

TABLE IV

Effect of Distance of Solvent Front on R_f Values

No.	Amino acids	With equilibration		
		Distance of the solvent front from the centre of the paper		
		5 cm.	10 cm.	15 cm.
1	Alanine	·55	·67	·55
2	α-Amino butyric acid ..	·53	·63	·59
3	Asparagine	·36	·45	·40
4	Aspartic acid ..	·40	·48	·45
5	Arginine	·40	·48	·45
6	Cystine	·25	·31	·26
7	Glycine	·45	·56	·43
8	Histidine	·34	·45	·43
9	Iso-leucine	·75	·84	·78
10	Leucine	·76	·82	·80
11	Lysine	·30	·40	·40
12	Methionine	·64	·78	·65
13	Ornithine	·30	·35	·32
14	Phenyl alanine ..	·70	·80	·79

The values are the average of triplicate experiments.

the solvent boundary from 5 cm. to 10 cm. and later decrease with increase of the distance from 10 cm. to 15 cm. from the centre.

The change in R_f values with increase in the distance of the solvent travelled may be attributed to the change in composition of the developer as it travels further. In the case of *n*-butanol-acetic acid-water solvent mixture the acid and probably water will be adsorbed preferentially from the mixture by the paper. The preferential adsorption of the acid from the solvent mixture at the centre of the paper was indicated by spraying the chromatogram after development with bromo-cresol-green. The central portion of the filter-paper was coloured yellow while the remaining portion was coloured green. Thus two zones were formed, the inner zone containing the acid adsorbed by the paper. Thus the solvent mixture was progressively depleted of the acid as it passed along the paper. This may affect the movement of the amino acids in the final stage resulting in the change in their R_f values as observed in these experiments. As the movement of the amino acids is considerably lower in butanol-water than in butanol-acetic

acid-water mixture, the lowering of the R_f values after the solvent had travelled greater distance may be attributed to the partial retention of the acid in the solvent mixture at the early stage during the movement of the solvent. Burma and Banerjee (1951) using *iso*-propyl alcohol-water (8·2) as solvent observed the R_f values of the amino acids, phenyl alanine and threonine remained constant irrespective of the distance run by the solvent. On the other hand, Kowkabany and Cassidy (1952) observed that the greater the distance over which the amino acid (valine) zone is developed the greater the R_f value up to a point. A more detailed investigation is necessary in order to throw more light on the relation between the movement of the amino acids and the distance of the solvent movement.

Effect of Distance of Initial Spot from the Centre of the Paper on R_f Value

Consden, *et al.* (1944) observed that the R_f values of amino acids changed with the distance from the developer surface at which the spot was initially placed. Burma (1951) also observed that the R_f values of the amino acids gradually decreased with the increase of the distance of their starting point from the centre of the solvent. This variation in R_f values was attributed to the gradual loss of water content of the solvent during the movement along the paper. Recently Kowkabany and Cassidy (1952) investigated the effect on R_f values of distance of initial spot from the surface of the developer and found that with the increase in distance of the spot from the surface of the developer the R_f values of the amino acid valine decreased. In view of the importance of the distance factor on the reproducibility of R_f values of amino acids, experiments were carried out on the variation in R_f values of amino acids with the increase in distance of the initial spot from the centre of the filter-paper as determined by circular paper chromatographic technique.

Concentric circles of varying radii (the difference in radius between two concentric circles being about 1 cm.) were drawn with a pencil from the centre of a filter-paper (18·5 cm. diameter). The amino acid solutions were spotted on the circumference of each circle, with one single spot on each concentric circle leaving a small space between two consecutive spots. After drying, the paper was irrigated with butanol-acetic acid-water solvent mixture and the development of the chromatogram was allowed to proceed until the solvent had travelled 9 cm. from the centre. After development the paper was dried and sprayed with ninhydrin reagent. The distance of the initial spot from the centre and the R_f values were measured and tabulated in Table V.

TABLE V

Effect of Distance of Original Spot from the Centre on R_f Values

(Distance of solvent boundary from the centre – 9 cm.)

No.	Amino acids	Distance of original spot from the centre (R_f values)							
		1 cm.	2 cm.	3 cm.	4 cm.	5 cm.	6 cm.	7 cm.	8 cm.
1	Amino-butyric acid ..	..	·65	·60	·56	·52	·48	·40	·20
2	Asparagine	..	·43	·40	·34	·26	·20	..	..
3	Aspartic acid	..	·50	·48	·40	·32	·30	·23	..
4	Arginine	..	·45	·43	·37	·34	·20	·20	..
5	Cystine	..	·33	·29	·20	·20	·10	..	..
6	Glutamic acid	..	·52	·45	·42	·34	·30	·20	..
7	Glycine	..	·50	·43	·37	·30	·20	·10	..
8	Iso-leucine	..	·79	·77	·75	·70	·66	·64	·60
9	Methionine	..	·65	·60	·55	·48	·42	·40	·35
10	Ornithine	..	·44	·40	·34	·25	·20	·10	..
11	Phenyl alanine	..	·70	·67	·64	·62	·56	·41	·40
12	Tyrosine	..	·65	·62	·59	·50	·45	·40	·30
13	Valine	..	·64	·62	·58	·52	·50	·50	·33

The values are the average of triplicate experiments.

The results show that the R_f values of all amino acids decrease with increase in the distance of the initial spot from the centre of the paper. This variation in R_f is due to the adsorption of acid and water present in the solvent as it travels, thereby the mobile phase is gradually depleted of its acid and water content resulting in the lowering of the R_f values. It is, therefore, desirable to fix the distance of the solvent boundary and the initial spot from the centre for each experiment for obtaining comparable and reproducible R_f values.

EFFECT OF TEMPERATURE ON R_f VALUE

Since the solubility of water in the solvent is influenced by temperature depending on the type of solvent mixture used, the R_f values also are affected by temperature, as the concentration of water in the solvent influences the rate of movement of the amino acids in the solvent mixture. In the case of solvents saturated with water the movement of amino acids is considerably influenced by temperature if the solubility of water in the solvent is altered by temperature. Thompson, *et al.* (1951) have shown that amino acids move more rapidly in the collidine-lutidine phase at low temperatures, as the solvent holds more water, while the effect of temperature is

not marked on the rate of movement in the phenol phase, as the effect of temperature on the solubility of water in phenol phase is small.

The effect of temperature on the movement of amino acids in *n*-butanol-acetic acid-water solvent mixture was investigated and the results are presented in Table VI.

TABLE VI

Effect of Temperature on the R_f Values of Amino Acids

Distance of solvent boundary from the centre—6 cm.

No.	Amino acids	6° C.	28° C.
1	Glutamic acid	·45	·46
2	Glycine	·39	·40
3	Histidine	·34	·32
4	Leucine	·75	·76

The data presented in the table show that the R_f values of the four amino acids investigated are not altered by increasing the temperature.

R_f VALUES OF SOME PEPTIDES

The R_f values of some peptides are presented in Table VII.

TABLE VII

R_f Values of Peptides

Solvent	Butanol : Water
Paper	Whatman No. 1
Distance of solvent boundary from the centre ..	9 cm.
Concentration of peptides used	0·2%.

No.	Peptides	R_f values
1	Alanyl Glycine	·19
2	Glycyl Glycine	·15
3	Glycyl Leucine	·45
4	Glycyl Tryptophan	·36
5	Leucyl-Glycyl-Glycine	·34

The chromatograms were run with *n*-butanol-water mixture, as the use of *n*-butanol-acetic acid-water as solvent was found to slightly hydrolyse some of the peptides.

The results show that the rates of movement of the peptides on the chromatogram is intermediate between the rates of movement of the constituent amino acids. These results are in conformity with the observations made by Hanes. *et al.* (1952).

Discussion

The results presented in Table I show that the R_f values of amino acids obtained by the circular paper chromatographic technique, using various solvents, are in general higher than those obtained by other investigators employing ascending and descending techniques. Some variation of R_f values of a given amino acid occurred from time to time and the values therefore, are subjected to $\pm$ 10 per cent. variation. Although the R_f values were found to vary with the conditions of a particular experiment, the relative positions of the amino acid bands were found to be in the same order for a particular solvent.

Among the solvents investigated, *n*-butanol-acetic acid-water mixture (40 : 10 : 50v/v) recommended by Partridge (1947) for sugars was specially suitable for the separation of amino acids. Clear and discrete bands, when mixtures of amino acids were used, could not be achieved by the use of any other solvent. In our experience, when phenol was used as solvent, it was not possible to obtain clear-cut separation of amino acids.

The R_f values of the amino acids in mesityl oxide were more or less in the same order as in butanol-acetic acid-water with a few exceptions. For example the R_f values of tyrosine and tryptophan are higher than that of valine, while in butanol-acetic acid-water the values are lower than that of valine.

With aqueous acetone as solvent the R_f values could be altered considerably by varying the percentage of water. In general, the addition of a larger proportion of water to acetone caused the amino acid to travel more rapidly on the chromatogram.

The results obtained in the present investigation as well as those obtained by others, indicate that adsorption as well as partition are involved in the movement of substances on paper. Consden, *et al.* (1944) postulate the view that adsorption of the amino acids by the cellulose plays no significant part and suggested partition mechanism for the separation of amino acids on paper. According to their view, the partition of the amino acid

on paper is determined by its partition coefficient between the mobile phase (the developing solvent mixture) and the stationary phase (water phase supported in the cellulose fibres). Hanes and Isherwood (1949) and Bentley and Whitehead (1950) postulated the view that simple partition as suggested by Consden, *et al.* (1944) is not the only factor involved in the separation of substances by paper chromatography. Hanes and Isherwood (1949) have developed a concept of the mechanism of paper chromatography. They consider that the water held by the paper is not free water but exists in a bound form, possibly linked together the chains of water molecules and the hydrophylic hydroxyl groups of the cellulose by hydrogen bonding, resulting in the formation of organised water cellulose complex which is not mobile, but existing in a tightly bound form. The water cellulose complex is considered to serve as the second phase rather than water and the affinity for water, therefore, depends on the water content of this complex and increases with decrease in the water content. The water content of the complex in equilibrium with different solvents, therefore, varies considerably and depends upon their affinities for water. Based on this behaviour of water cellulose complex Hanes and Isherwood state, " The problem of the mechanism of the chromatogram centres on how this water-cellulose complex holds solute molecules in competition with the flowing solvent." They suggest that a solute molecule, by virtue of its hydrophilic nature, will compete with water and solvent molecules, which also contain hydrophilic groups in the mobile phase for incorporation in the cellulose-water complex. The separation of the solutes, therefore, depends on the difference in their distribution between the two phases, the distribution, in turn, depending on the extent to which it is incorporated into the water-cellulose complex, on the one hand, and the partial organisation of water characterising the flowing solvent on the other. The R_f values (distribution of the solvent between the two phases) will depend on such factors as size and shape of molecule, number, position and character of the hydrophilic groups of the solute and the solvent and also on the water content. The R_f values will, therefore, be influenced by the change in composition of the solvent.

In support of this hypothesis, Bentley and Whitehead (1950) have shown that the R_f values of the amino acids investigated increase with the increase in hydrophilic character of the alcohols used as solvents. The increase in hydrophilic character of the solvents results in greater competition by the alcohol in preference to solute molecules for incorporation in the cellulose-water complex, resulting in the greater movement of the solute in the mobile phase.

A mechanism such as this is necessary to explain the movement of amino acids on paper when water-miscible solvents such as acetone are used as developing solvents.

Note added in Proof.—While correcting this proof, a paper by P. S. Rao and R. M. Beri (*Proc. Ind. Acad. Sci.*, 1952, **36**, 370) appeared, in which the authors report the results on the R_f values of amino acids in various solvents by Circular Paper Chromatography and state "The values now obtained are quite characteristic and reproducible, and are much different from the straight R_f values and this method may offer a convenient, quick and facile way of identifying the amino acids". Except for the statement that the R_f values obtained by Circular Paper Chromatography are different from those obtained by the ascending and descending techniques, which is in conformity with the observations recorded in this paper, we disagree with the views of these authors. An examination of the R_f values given by Rao and Beri indicate that some of the values reported are neither characteristic nor reproducible. The R_f values given by Rao and Beri for cystine (0·60), glutamic acid (0·49–0·60), arginine (0·51), and histidine (0·52) do not indicate the correct position of these amino acids on the chromatogram developed with Butanol-acetic acid-water as solvent mixture. On the basis of these R_f values, the cystine band should come above the glutamic acid band which is incorrect and contrary to our own observations and those of other workers in the field. Cystine always takes the lowest position on the chromatogram when Butanol-acetic acid-water is used as solvent mixture, and hence the R_f value of this amino acid should be very much lower than glutamic acid. Similar discrepancies may be noted in their R_f values reported for other amino acids. The reproduction of R_f values exactly is very difficult as the values are influenced by various factors, such as type of paper, temperature, the nature of the solvent, the distance of the solvent front, pH, etc. It also varies from batch to batch of paper used and as such identification of amino acids by R_f values alone without running simultaneous controls with known amino acids on the same paper is not reliable. This view is also expressed by other workers in the field. Mixed chromatograms by running simultaneous controls with known amino acids for identification of the unknown can only be carried out by adopting the technique described by Giri and Rao in Part I of this series of publication, and in this technique the use of a detachable 'wick' is advantageous and convenient. Although Rao and Beri state that "the provision of a detachable tail or 'wick' for irrigating the filter-paper with the solvent, as suggested by Giri was not found to be of any special advantage", recently several workers have found it very

convenient and have adopted this technique in their investigations on the separation and identification of amino acids and other substances by circular paper chromatography.

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