CHROMATOGRAPHIC METHODS FOR THE IDENTIFICATION OF BIOGENIC AMINES*

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The term "Biogenen Amine" was first used by GUGGENHEIM in 1920, to describe a large group of compounds with basic properties, occurring naturally and possessing some properties in common. Many methods have been used for their isolation and identification. No systematic methods have been developed to identify them as a group. In animals and most bacteria, amines are usually the product of decarboxylation of amino acids and/or their derivatives. This limits the number of basic compounds that would occur naturally. Several methods have been reported for the separation of known amines by means of paper chromatography[1,2]. Direct chromatography of tissue extracts would be unsatisfactory since the concentration of amino acids in tissues is greater than the concentration of amines. This would mask any amines present in small amounts; also the amines would be indistinguishable from amino acids by direct development of color with ninhydrin. Weakly acidic cation exchange resins have been used in special cases. Amberlite IRC-50 has been used to separate adrenaline from nor-adrenaline[3-5] and also to separate some bases from urine[6]. It has been used in columns for the chromatography of basic amino acids[7]. In our laboratory we succeeded in separating basic amino acids and few amines using columns of buffered Amberlite IRC-50 and gradient elution. Buffers of increasing molarity were used, but the results were not good. Many amines failed to develop sharp boundaries and others decomposed in the presence of large amounts of salts. We abandoned this method in favor of partition chromatography on cellulose columns. Good separations were obtained in a few trial runs. In order to use cellulose as the stationary phase it was necessary to find a method to separate the amines from amino acids prior to chromatography and to obtain the solution of amines free of salts. We have succeeded in obtaining such solutions.

The methods reported here are methods for the quantitative separation of amines from amino acids and for the fractionation of amines by partition chromatography on cellulose columns and filter paper.

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MATERIALS AND METHODS

Reference compounds

Amino acids and amines used were all obtained from the California Corporation for Biochemical Research in the best grades available.

Resin

Amberlite CG-50, Type 2, with a screen grading of approximately 200 (passing 200 mesh) was used. This resin was purified according to the procedure described by HIRS, MOORE AND STEIN for the purification of Amberlite IRC-50[8]. (This is the same type resin as CG-50 but of a different grade.) When the purification was completed, the resin was converted to the hydrogen form and stored.

Powder cellulose

The powder cellulose was obtained from Brown Company, Boston, and is sold under the name of Solka-Floc. The cellulose was obtained with a screen grading of 200 mesh. Before use the powder cellulose was purified by washing several times with 1 *N* sodium hydroxide and 1 *N* hydrochloric acid. The cellulose was washed with distilled water and afterwards with alcohol. The cellulose was then dried by placing it under infrared lamps.

Detection of fractions

The fractions obtained from each column were analyzed by determining their absorbancy at 279 mμ with a Beckman Model DU Spectrophotometer. An aliquot was removed from each fraction and analyzed by the ninhydrin method of MOORE AND STEIN[9]. Other aliquots were taken and analyzed by paper chromatography. The papers were treated with (a) ninhydrin and (b) diazotized sulfanilic acid.

EXPERIMENTAL RESULTS

1. *Separation of mono- and dicarboxylic amino acids from amines and basic amino acids*

One of the difficulties in detecting small amounts of amines in tissues is that they are indistinguishable from amino acids by many of the known tests. Extraction of amines by solvents has disadvantages; one must operate at extremely alkaline pH and solvent extraction is seldom specific for one group of compounds. To obtain the amines from tissues free of amino acids, we tried several methods. WINTERS AND KUNIN[10] suggested a scheme for the separation of basic amino acids by means of Amberlite IRC-50 buffered to pH 4.7. We tried the same method to separate amines from amino acids. The separation was complete but the amine fraction was contaminated with salts eluted from the resin. Amberlite IRC-50, being a carboxylic type resin, reaches equilibrium only slowly when operated in the acid form. To obtain an amine fraction free of salts we had to use the resin in the acid form. In order to attain equilibrium the

resin was allowed to remain in contact with the solution for one hour and with constant shaking. We finally decided on the following method. An amount of purified resin was added to the mixture of amino acids and amines. The amount used was in excess of the amount needed to reach saturation of the resin. After shaking the mixture in a mechanical shaker for one hour, the resin and solution were poured in a 1 × 15 cm column containing 1 g of the resin. After the resin had settled, the column was washed with water until all the amino acids were washed out of the column. The resin containing the amines and the basic amino acids was eluted with 2 × 10 ml of 4 *N* acetic acid. Recovery experiments were carried out using individual mono-carboxylic amino acids and a number of amines. The results are shown in Table I. It is clear from

TABLE I

RECOVERY OF AMINO ACIDS FROM COLUMNS OF AMBERLITE CG-50 AND RETENTION OF AMINES

Compound added 5 μmoles	*Found in effluent μmoles*	*Eluted from resin μmoles*	*% Recovery*
Glycine	5.22	—	104.4
Alanine	5.18	—	105.6
Threonine	5.04	—	100.8
Serine	5.52	—	110.0
γ-Aminobutyric acid	5.02	—	100.4
Leucine	4.84	—	96.8
Phenylalanine	5.20	—	104.0
Tyrosine	5.02	—	100.4
Tryptophan	4.65	—	93.0
Arginine	—	5.21	104.2
Lysine	—	5.00	100.0
Histidine	—	5.00	100.0
Phenylethylamine	—	5.53	111.0
Tryptamine	—	5.03	100.6
Tyramine	—	4.94	98.8
Serotonin	—	5.02	100.4
Epinephrine	—	5.07	101.4
Norepinephrine	—	4.79	95.8
Hordenine	—	5.07	101.4
3-Hydroxytyramine	—	4.97	99.4

Table I that mono-carboxylic amino acids are not retained by the resin and that they can be recovered quantitatively in the effluent. It is also noted that the amines are retained quantitatively and eluted quantitatively from the resin bed. The analyses of both amines and amino acids were carried out by ultraviolet spectrophotometry and by the ninhydrin method. Verification of the degree of fractionation was possible by paper chromatography. By this method the separation is complete and quantitative. In Table II we show that a mixture of an amino acid and its corresponding amine can be separated quantitatively.

2. *Column chromatography*

A number of preliminary experiments were carried out to find the best solvents for fractionating amines. Mixtures of several reference amines were chromatographed on

TABLE II

SEPARATION OF AMINO ACIDS AND AMINES WITH AMBERLITE CG-50

Added to resin 5 μmoles each	*Recovered*			
	by ninhydrin method		*by ultraviolet absorption*	
	μmoles	*%*	*μmoles*	*%*
Tyrosine	4.66	93.2	5.04	100.8
Tyramine	4.71	94.2	5.19	103.8
5-Hydroxytryptophan	5.44	108.8	5.31	106.2
Serotonin	4.82	96.4	5.05	101.0
Tryptophan	5.31	106.2	4.92	98.4
Tryptamine	5.28	106.6	5.17	103.4
Phenylalanine	5.47	109.4	—	—
Phenylethylamine	5.28	105.6	—	—

filter paper to determine their R_F values in several solvents. Of the solvents tried butanol–acetic acid–water in a 4:1:1 proportion and 4:1:1.6 proportion gave very good results. Best results were obtained with 2-butanone–propionic acid–water in a 3:1:1.2 proportion. Unfortunately this solvent mixture cannot be used in columns although it gives excellent results on paper. In columns there are many disadvantages; the presence of the ketone interfered with the detection of compounds absorbing ultraviolet light. Too, the development of the ninhydrin color is inhibited. We used this solvent to verify the homogeneity of the fractions obtained from columns.

The procedure used for the chromatography of known mixtures of amines was the same every time. The columns were packed by several additions of a suspension of cellulose powder in solvent. Occasionally slight pressure was applied. The solvent to be used was allowed to wash the column until the effluent gave a low and constant reading when read at 279 mμ and no color when tested with the ninhydrin reagent. When the column was ready the mixture of amines was pipetted, usually in a 1 ml volume, made up with the chromatographic solvent. The solvent was then allowed to flow at a rate of about 4 ml per hour. Fractions of 1 ml volume were collected. Each fraction was analyzed as follows: (a) absorption at 279 mμ, (b) color produced with ninhydrin, and (c) chromatography on filter paper with a solvent different from that used on the column.

(*A*) *Fractionation with butanol–acetic acid–water, 4:1:1.* This solvent gave good separation of several amines on filter paper. A mixture of 2 μmoles of each of the following amines was prepared: valamine, phenylethylamine, tryptamine, tyramine, serotonin, 3-hydroxy-tyramine, epinephrine, norepinephrine, ethanolamine, histamine, cadaverine, putrescine, arginine, lysine, threamine. The mixture was chromatographed on a column as previously described. The results obtained are shown in Fig. 1.

(*B*) *Fractionation with butanol–acetic acid–water, 4:1:1.6.* The results obtained with the mixture previously described are satisfactory for some of the amines. The aromatic compounds separate well but the more polar substances tend to be retarded and as a result of this retardation the boundaries become distorted. We thought that by increasing the polarity of the solvent slightly we would increase the

rate of migration of the more polar substances without affecting materially the less polar substances. For this we chose the solvent mixture of butanol–acetic acid–water in a proportion of 4:1:1.6. The results obtained with this solvent using the same

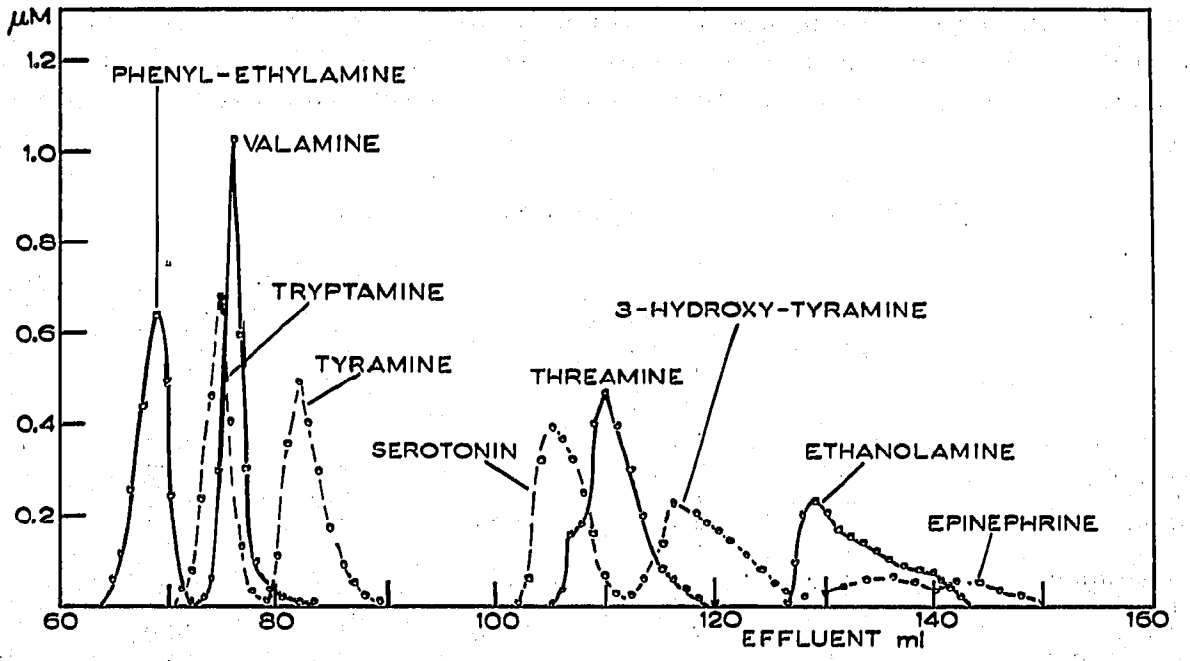

Fig. 1. Chromatographic separation of a mixture of amines on a cellulose column (80 × 1 cm). Solvent: butanol–acetic acid–water, 4:1:1. Rate of flow: 4 ml per hour. Solid line represents concentration of effluent measured by the ninhydrin method. Broken lines represent concentration measured by absorption at 279 mμ.

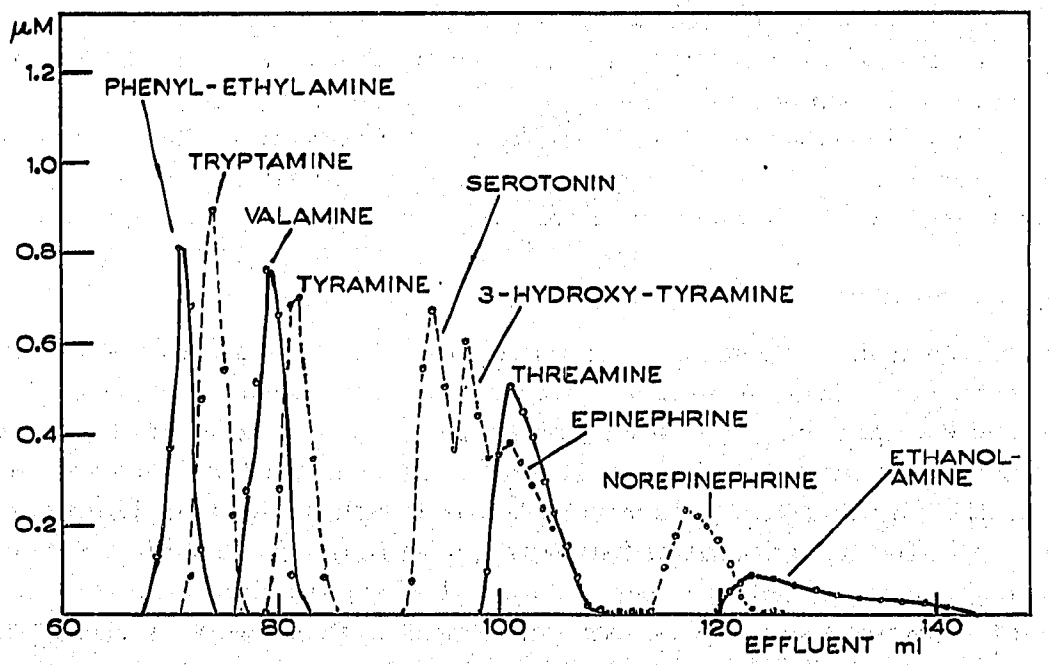

Fig. 2. Chromatographic separation of a mixture of amines in a cellulose column (80 × 1 cm). Solvent: butanol–acetic acid–water, 4:1:1.6. Conditions were the same as those described in Fig. 1.

compounds as those used in the previous column are shown in Fig. 2. The higher polarity of this solvent over the previous one causes most compounds to move faster. In the less polar solvent several amines and the basic amino acids failed to yield clear-cut boundaries. No separation of them was accomplished. With the more polar mixture, the epinephrine and norepinephrine bands were sharper and the recoveries considerably better than in the less polar solvent (Table III). It is difficult to decide which is the better solvent. For some aromatic amines the less polar solvent is definitely better.

TABLE III

RECOVERY OF AMINES FROM CELLULOSE COLUMNS

Compound	*Solvent*					
	Butanol–AcOH–H_2O (4:1:1)			*Butanol–AcOH–H_2O (4:1:1.6)*		
	added µmoles	*recovered µmoles*	*recovery %*	*added µmoles*	*recovered µmoles*	*recovery %*
Phenylethylamine	2.0	2.14	107.0	2.0	2.17	108.5
Tryptamine	2.0	1.96	98.0	2.0	2.07	103.5
Valamine	2.0	1.97	98.5	2.0	1.85	92.5
Tyramine	2.0	2.18	109.0	2.0	2.12	106.0
Serotonin	2.0	1.95	97.5	2.0	2.30	115.0
3-OH-tyramine	2.0	1.96	98.0	2.0	2.10	105.0
Epinephrine	2.0	0.98	49.0	2.0	2.01	100.5
Threamine	2.0	2.29	114.5	2.0	2.28	114.0
Norepinephrine	2.0			2.0	1.22	61.0
Ethanolamine	2.0	1.98	99.0	2.0	0.86	43.0

(*C*) *Fractionation with 2-butanone–propionic acid–water, 3:1:1.2.* This solvent gave the best results on paper chromatography. We tested it as a possible solvent for columnar separation even though the analysis of the fractions cannot be carried out by the same means as those described. The fractions were analyzed by paper chromatography using butanol–acetic acid–water mixtures for solvents. The results are plotted in a different form since there is no quantitative data available. In Fig. 3 one can see that the separation with this solvent is the best.

3. *Paper chromatography*

Paper chromatography of amines and basic amino acids has not been satisfactory. As an adjunct to columnar separation, paper chromatography is extremely valuable. Whatman 3 MM paper was used in all instances. The best solvents found were those described for the columns. Chromatography was carried out by the ascending method. After drying the papers at 40°, they were treated with ninhydrin. Duplicate chromatograms were treated with diazotized sulfanilic acid to detect any phenolic compounds. In Fig. 4 are shown some selected separations. The separation is not only satisfactory but the spots obtained are compact and well defined. Of the solvents used 2-butanone–propionic acid–water was the best. We have tried a number of solvents containing ammonia but the results were not good. In many cases there was poor definition of

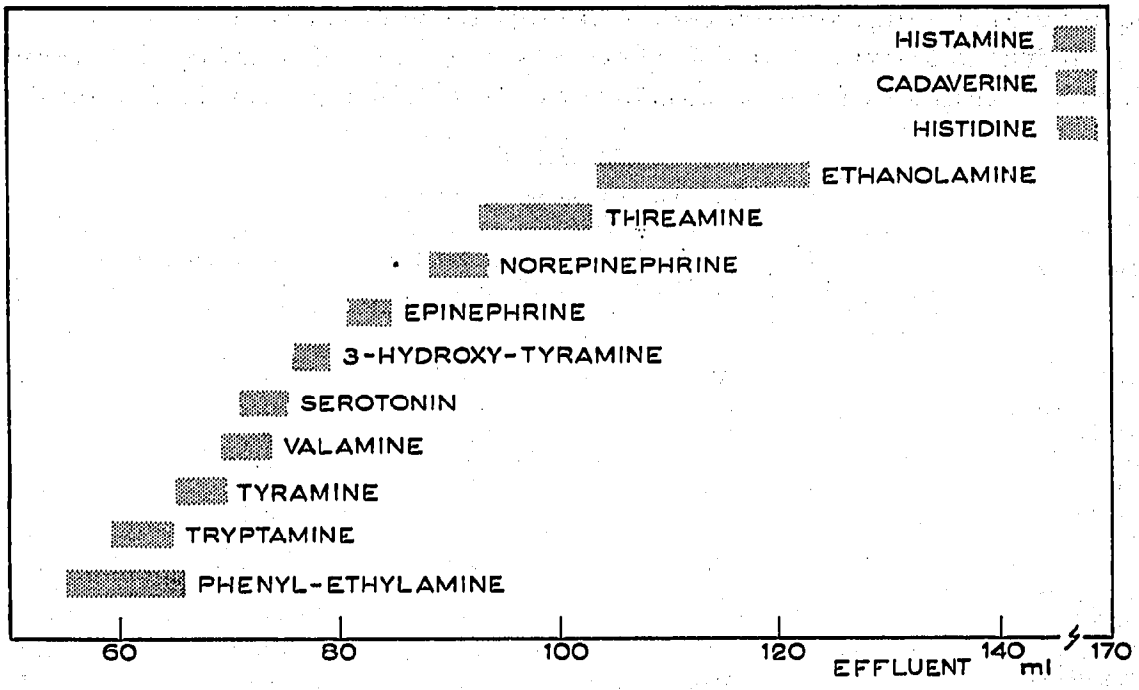

Fig. 3. Chromatographic separation of a mixture of amines on a cellulose column (80 × 1 cm). Solvent: 2-butanone–propionic acid–water, 3:1:1.2. Analyses of fractions carried out by paper chromatography. Each bar represents the volume of effluent in which the fraction was found.

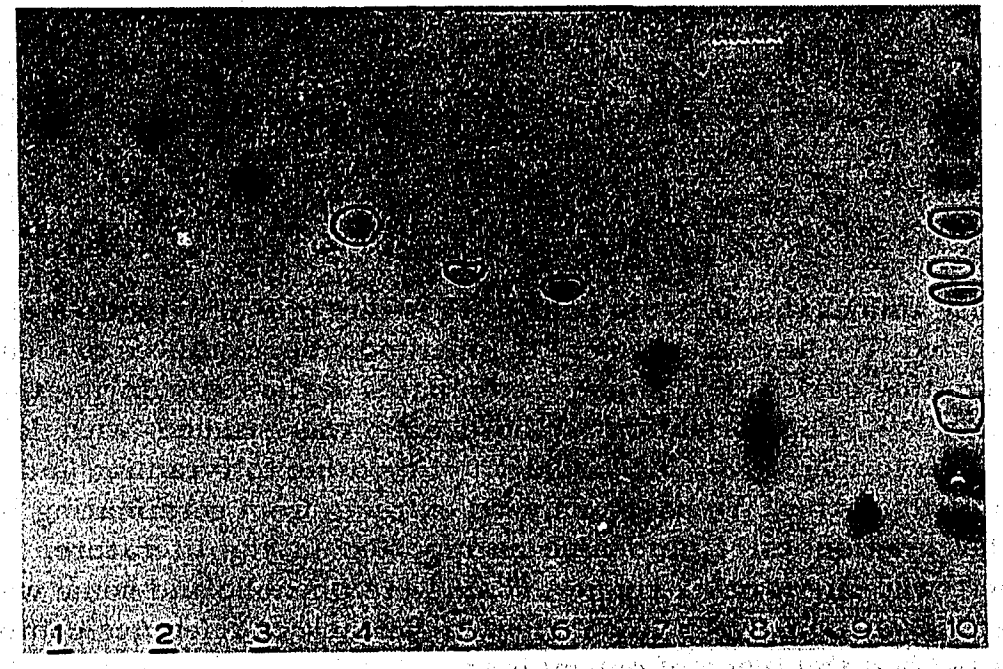

Fig. 4. Chromatographic separation of a mixture of amines on filter paper (Whatman 3 MM). Solvent: 2-butanone–propionic acid–water, 3:1:1.2. Spots marked with pencil give colors that do not show well in photographs. (1) Phenylethyl amine, (2) Tryptamine, (3) Tyramine, (4) Serotonin, (5) Epinephrine, (6) Norepinephrine, (7) Histamine, (8) Putrescine, (9) Lysine, and (10) mixture of all of them.

boundaries and trailing. Many phenolic amines are very unstable in alkaline solutions. Butanol–acetic acid–water mixtures were satisfactory for many compounds. The R_F values of a number of compounds in these mixtures are given in Table IV.

TABLE IV

R_F VALUES OF SOME AMINES

Compound	*2-Butanone–propionic acid–H_2O 75:25:30*	*Butanol–AcOH–H_2O 120:30:30*	*Butanol–AcOH–H_2O 120:30:40*	*Butanol–AcOH–H_2O 120:30:50*
Norleucamine	0.92	0.80	0.79	0.80
Leucamine	0.91	0.81	0.77	0.81
Phenylethylamine	0.88	0.69	0.79	0.81
Norvalamine	0.87	0.77	0.71	0.76
Hordenine	—	0.63	0.77	—
Valamine	0.86	0.66	0.74	0.75
Tryptamine	0.86	0.60	0.67	0.73
Tyramine	0.77	0.58	0.59	0.67
Serotonin	0.64	0.38	0.41	0.47
3-OH-tyramine	0.58	0.41	0.39	0.46
Threamine	0.55	0.43	0.43	0.47
Epinephrine	0.54	0.30	0.32	0.45
Norepinephrine	0.48	0.26	0.28	0.40
Seramine	0.46	0.34	0.34	0.38
Agmatine	0.46	0.21	0.20	0.34
Histamine	0.43	0.20	0.20	0.30
Cadaverine	0.39	0.16	0.14	0.26
Putrescine	0.32	0.13	0.11	0.20
Arginine	0.26	0.09	0.04	0.17
Glutamine	0.25	0.11	0.16	0.25
Lysine	0.24	0.06	0.07	0.14
Histidine	0.23	0.09	0.08	0.16
Carnosine	0.23	0.08	0.08	0.15
Methyl-histidine	0.23	0.08	0.10	0.18
Thiohistidine	0.14	0.11	0.08	0.18
Spermine	0.05	—	—	—

POSSIBLE APPLICATIONS

There are many amines that occur in nature. There are probably many more that have not been detected. We have tried to use the methods reported here to identify hordenine in germinating barley; this amine accumulates in the roots during the first week of germination[11,12]. The formation of hordenine was studied with labeled compounds[13–15]. It is formed by decarboxylation of tyrosine to tyramine and subsequent methylation of the latter. We have analyzed extracts from germinating barley roots and found large amounts of hordenine as reported in the literature. In addition, however, there were eight other amines; some were phenolic and some were not. We have not carried out this work to completion for lack of reference compounds and because it is beyond the scope of our interest.

As a possible extension of this work, we believe that one can develop a relatively easy method for measuring amino acid decarboxylases. The separation of an amine and its parent amino acid is complete. Thus, one can remove the excess substrate by

means of Amberlite CG-50 after enzymic action, and measure the amine formed by any suitable method. Many methods cannot distinguish between the amine and the parent amino acid. In dealing with the amine only, the number of applicable methods to its measurement is increased several times.

SUMMARY

1. Column chromatography in which the stationary phase is cellulose and the mobile phase is a mixture of butanol–acetic acid–water is reported as a method to separate a number of amines. These amines either occur naturally or are likely to occur.

2. Paper chromatography in which similar solvents were used and also 2-butanone–propionic acid–water is reported as a method to separate amines and a number of amino acids and derivatives. The R_F values in these solvents are given for 26 compounds studied.

3. A method to separate quantitatively amines from amino acids (except the basic amino acids) is reported. The possibility of using this method for the measurement of some amino acid decarboxylases is discussed.

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