6. Although the calculated values of ionic diffusion coefficients are not exact, the uncertainties in these values are relatively small.

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[CONTRIBUTION FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

Chromatography as a Means of Separating Amino Acids[1]

BY JACQUES L. WACHTEL[2] AND HAROLD G. CASSIDY

The separation of several binary mixtures of amino acids was reported in a preliminary paper from this Laboratory.[3] The method of separation used was that of Tswett-column adsorption analysis[4] employing a special brand of commercial charcoal as the adsorbent. This method has now been extended to the separation of a quaternary mixture of amino acids, and this separation will be described, giving full details of the method. It is hoped to extend the method to the separation of more complex mixtures.

Other investigators have used adsorption analysis for the separation of amino acids. Whitehorn[5] and Felix and Lang[6] used permutit for the separation of the basic amino acids from the neutral and the acidic amino acids. Johnson[7] separated glutamic acid from histidine on adsorption columns of titania gel. Tiselius,[8] using a modified Tswett-column technique in combination with the Toepler schlieren method for following changes in refractive index, analyzed a mixture of alanine, valine and leucine. Martin and Synge[9] described the separation of the acetyl derivatives of certain amino acids using a "chromatogram" employing two immiscible liquid phases one of which was supported on a solid phase.[10]

Materials.—**Solvents** were distilled in all glass apparatus to remove non-volatile material. The **adsorbent** used was Darco G-60, which was obtained from the Darco Corp., New York City. **Glycine.**—Eastman Kodak Company ammonia-free glycine was decolorized, recrystallized from water and vacuum dried at 90°. Nitrogen, by Kjeldahl, found, 18.68, 18.70% (calcd. 18.67%). ***dl*-Leucine.**—The *dl*-leucine was prepared from crude material by decolorization and recrystallization from aqueous alcohol. Nitrogen, found, 10.60, 10.70% (calcd. 10.68%). A sodium fusion test for sulfur was negative, indicating the absence of methionine. A 5% solution in 10% hydrochloric acid showed no optical rotation. ***dl*-Phenylalanine.**—A synthetic preparation was decolorized and recrystallized three times from aqueous alcohol. No satisfactory Kjeldahl analyses could be obtained either with this material or with other samples of synthetic *dl*-phenylalanine obtained from other sources. The hydrochloride made from this material was found to contain 17.60% chlorine (av. of four determinations, calcd. 17.58%). Van Slyke amino nitrogen gave 8.49, 8.55% (calcd. 8.49%). A sample, 1.010 milliequivalents, was titrated with alkali using a glass electrode to follow the changes in the *p*H.[11] The titration data gave a value of 1.009 milliequivalents. ***l*-Tyrosine.**—*l*-Tyrosine from Paragon Testing Laboratories was decolorized in hydrochloric acid solution, precipitated, washed and recrystallized from boiling water. Kjeldahl nitrogen determinations gave 7.69, 7.75% (calcd. 7.74%). The purified material gave a negative nitroprusside test for cystine. A 5% solution in 4% hydrochloric acid showed $[\alpha]^{29}D$ −7.40°.

(1) Material for this paper was taken from the dissertation submitted by J. L. Wachtel to the faculty of the Graduate School, Yale University, 1942, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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(3) J. Wachtel and H. G. Cassidy, *Science*, **95**, 233 (1942).

(4) L. Zechmeister and L. Cholnoky, "Principles and Practice of Chromatography," John Wiley and Sons, Inc., New York, N. Y., 1941; H. H. Strain, "Chromatographic Adsorption Analysis," Interscience Publishers, Inc., New York, N. Y., 1942.

(5) J. C. Whitehorn, *J. Biol. Chem.*, **56**, 751 (1923).

(6) K. Felix and A. Lang, *Z. physiol. Chem.*, **182**, 125 (1929).

(7) Sr. M. B. Johnson, Dissertation, Catholic University of America, Washington, 1938.

(8) A. Tiselius, *Arkiv. Kemi, Mineral., Geol.*, **14B**, #22 (1940); **14B**, #32 (1941); **15B**, #6 (1941); *Science*, **94**, 145 (1941); "Advances in Colloid Science," ed. E. O. Kraemer, Interscience Publishers, Inc., New York, N. Y., 1942, pp. 81–97.

(9) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35**, 1358 (1941).

(10) It is unfortunate that Martin and Synge[9] have used the term "chromatography" to describe their new and ingenious method. The name chromatography has hitherto been used to describe processes involving countercurrent adsorption of the Tswett-column type. In a "chromatogram" of the Martin and Synge type separations "depend upon differences in the partition between two liquid phases of the substances to be separated, and not, as in all previously described chromatograms, on differences in adsorption between liquid and solid phases." The method of Martin and Synge is analogous to chromatography in the sense that all separation processes which depend upon partitions are analogous; however, it appears more nearly to be a special kind of countercurrent liquid-liquid extraction process, in which one liquid phase is held substantially stationary. Indeed this is indicated by their "Theory of Chromatography." For the purposes of this theory their partition coefficient is assumed to be linear. It is well known that the "partition coefficients" of adsorption processes are not linear. It is true that their "theory of chromatography" could be made to describe an adsorption partition by suitable modification of the partition coefficient, but as was mentioned above this is because of the general analogy between partition processes (see, for example, M. Randall and B. Longtin, *Ind. Eng. Chem.*, **30**, 1063 (1938)). It may be hoped that the term chromatography will not come into general use to describe the process of Martin and Synge, for this will lead to unnecessary confusion in terminology.

(11) See M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 359 (1936).

Experimental.—The separation of a mixture of glycine, *dl*-leucine, *dl*-phenylalanine and *l*-tyrosine will be described. Separations of binary and ternary mixtures were carried out in a manner similar to that of the four-component mixture; indeed the four-component separation involved essentially a group separation followed by a pair of simpler separations, and these will therefore need no additional description.

The quaternary separation was made using about one-tenth the quantities of amino acid used in the simpler separations.[3] This was done for two reasons. First, it was pointed out by H. B. Vickery[12] that a method of separation applicable to small quantities of amino acids would be of value in the analysis of proteins which can be obtained only in small amounts. Second, it was hoped that by using smaller quantities of amino acids a decomposition observed during the course of the binary and ternary separations would be reduced in extent, since the time required for making the separation would be shortened.

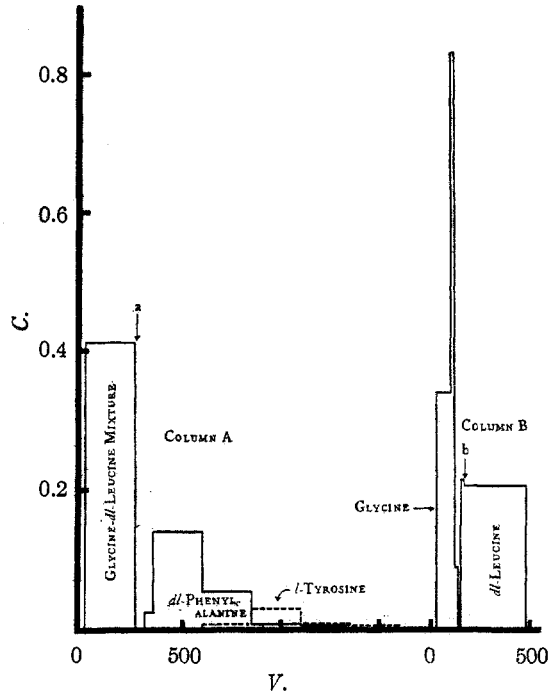

Fig. 1.—Separation of glycine, *dl*-leucine, *dl*-phenylalanine and *l*-tyrosine. *V*, volume of percolate in cc., collected in fractions as shown; *C*, concentration of percolate in mg. per cc. a, 5% acetone developer solution added at this point; b, 5% ethyl acetate eluant added at this point.

Two adsorption columns designated as A and B were used for the quaternary separation (see Table I and Fig. 1). The adsorbent mixture for Column A consisted of 1.4 g. of charcoal and 3 g. of pulped filter paper; that for Column B of 2.6 g. of charcoal and 4 g. of pulped filter paper.[13] The filter paper and the charcoal were each extracted three times with boiling water. They were then mixed by means of an egg-beater. Each adsorption column was formed in a glass tube about 2 cm. in internal diameter.

A mixture of 37.87 mg. of glycine, 66.52 mg. of *dl*-leucine, 58.25 mg. of *dl*-phenylalanine, and 10.12 mg. of *l*-tyrosine in 10 cc. of aqueous solution was applied to column A and developed with water.

Column A served the dual function of (a) separating (rapidly) the less well adsorbed glycine and leucine from the more strongly adsorbed phenylalanine and tyrosine, and (b) separating (more slowly) the phenylalanine from the tyrosine. The glycine and leucine were collected together in one fraction of percolate, the limits of which (see table and fig.) were marked by the absence of amino acid: that is, at first there was no glycine test and no ninhydrin test, and after collection of this fraction there was no ninhydrin test and no phenylalanine test. After the glycine and leucine had been washed from Column A the column was developed with 5% aqueous acetone until phenylalanine began to appear in the percolate. The column of adsorbate was then extruded and cut into 5 sections of equal length. Each of these was eluted with 225 cc. of freshly prepared 5% aqueous ethyl acetate, and the eluates made up to 250 cc. for analysis.

The mixed glycine–leucine fraction from Column A was concentrated *in vacuo* to about 20 cc. and applied to column B. A complete description of the progress of this separation is given in Table I.

The separations obtained with Column A required four hours; the separation of leucine and glycine on Column B, two and one-half hours. Each of these periods was much shorter than those required to make comparable separations using ten times as much of each amino acid.

The leucine fraction from Column B was triturated with dried and redistilled acetone to remove impurities eluted from the adsorbent along with the leucine by the aqueous ethyl acetate.[14] Glycine, phenylalanine and tyrosine were determined colorimetrically in an Evelyn colorimeter. Glycine was determined using the modified *o*-phthaldialdehyde method of Patton,[15] phenylalanine by the method of Kapeller-Adler,[16] and tyrosine by a modification of the method of Folin and Marenzi.[17] Fractions A-1, B-2, B-3, B-4, B-5 and B-7 were tested for ammonia by aeration into boric acid.[18] None was found. The entire amounts of

(12) Private communication.

(13) In previous separations the ratio of charcoal to filter paper pulp used was about 1:1. The ratio was changed to about 1:2 in this experiment so that the column of adsorbent mixture could be made large enough in diameter (about 2 cm.) to give a good rate of flow, and still be long enough to effect a good separation. It had previously been shown[3] that the filter paper pulp did not adsorb the amino acids. It was used as a bulking agent, and was prepared by boiling filter paper clippings with distilled water and then beating with a mechanical egg-beater.

(14) Purification of the adsorbent needs further investigation. In preliminary experiments Darco G-60 was washed exhaustively with 5% ethyl acetate and then re-activated *in vacuo* at about 400°. The purified and reactivated charcoal adsorbed leucine, phenylalanine and tyrosine to a slightly less extent than did the untreated adsorbent. Leucine and tyrosine could be separated on an adsorption column of this purified adsorbent. It remains to be found out if adsorption of amino acids on the purified charcoal might displace more material which would then become elutable by 5% ethyl acetate.

(15) A. R. Patton, *J. Biol. Chem.*, **108**, 267 (1935). For very small samples the method of W. Zimmerman, *Z. physiol. Chem.*, **189**, 4 (1930), was used.

(16) R. Kapeller-Adler, *Biochem. Z.*, **252**, 185 (1932).

(17) O. Folin and A. D. Marenzi, *J. Biol. Chem.*, **83**, 89 (1929). The steps taken to ensure removal of tryptophan were omitted.

(18) See, for example, N. M. Stover and R. B. Sandin, *Ind. Eng. Chem., Anal. Ed.*, **3**, 240 (1931).

fractions A-1, B-4, and B-5, were used for glycine analysis. The total weights of the glycine and leucine fractions were somewhat greater than the amounts of glycine and leucine used. This was probably due to inorganic material dissolved from the charcoal. (Small amounts of ash were found in fractions from larger-scale experiments.) The colorimetric analyses for glycine indicated a recovery of 37.89 mg. (37.87 mg. applied). The weights of the leucine fractions totaled 67.34 mg. (66.52 mg. applied). Colorimetric analyses indicated recoveries of 8.61 mg. of tyrosine (85.0%). and 49.10 mg. of phenylalanine (84.3%). Some of each of these amino acids was therefore lost during the separation.

Nitrogen (Kieldahl) analyses gave the following results. Fraction B-2 (glycine) 18.62, 18.79% (calcd. 18.67%).

TABLE I

SEPARATION OF GLYCINE, *dl*-LEUCINE, *dl*-PHENYLALANINE, AND *l*-TYROSINE

COLUMN A

Fraction	Vol., cc.	Remarks
A-1	20	No ninhydrin test, no glycine test, wt. 0.24 mg., disregarded.
A-2	250	Contained about 0.1052 mg. solid. Concentrated *in vacuo* to about 20 cc., applied to Column B.
		Development with 5% aqueous acetone was begun at this point.
A-3	52	No ninhydrin test, no phenylalanine test, wt. 0 93 mg., disregarded.
A-4	42	Wt. 2.31 mg., contained 0.835 mg. phenylalanine, colorimetrically.
		The column of adsorbate was extruded and cut into 5 sections of equal length. Each was eluted with 225 cc. of 5% ethyl acetate.

Fraction	Phenylalanine eluted determined colorimetrically, mg.	Tyrosine eluted detd. colorimetrically, mg.
A-5	34.42	None[a]
A-6	12.87	1.18
A-7	0.80	6.75
A-8	0.17	0.58
A-9	None[a]	0.10

COLUMN B[b] (Fraction A-2 was applied to this column, developed with water)

Fraction	Vol., cc.	Composition	Content
B-1	25	...	0.0 mg.
B-2	70	Glycine	23.67 mg. colorimetrically
B-3	15	Glycine	12.46 mg. colorimetrically
B-4	20	Glycine	1.675 mg. colorimetrically
B-5	15	No glycine test, no ninhydrin test.	
B-6	21.3	*dl*-Leucine	4.54 By weight (no glycine test).
		At this point 5% aqueous ethyl acetate was used as an eluant.	
B-7	310	*dl*-Leucine	62.80 By weight.

[a] "None" indicates no color test was obtained. [b] The amounts of leucine were taken as the weights of non-volatile material obtained after trituration with dry acetone (see above).

Fraction B-6 (leucine) 10.8% (calcd. 10.68%). Fraction B-7 (the main leucine fraction) 10.42, 10.42, 10.43%. Comparison of the nitrogen contents of these leucine fractions with those of the leucine fractions from a large-scale (and longer duration) separation of glycine and leucine revealed that if aminolysis of the leucine had occurred on columns A and B, its extent was greatly reduced.

Discussion.—It was observed during preliminary work (not previously reported) that some decomposition seemed to occur during the separations. There are apparently at least two types of decomposition of amino acids that can occur on activated carbon. One of these is a complete oxidation of the amino acid to carbon dioxide, water and ammonia. This type of decomposition has been studied by Warburg and Negelein.[19] The other type of decomposition involves the hydrolytic removal of the amino group (aminolysis) from the amino acid, giving rise principally to the corresponding hydroxy acid. The aminolysis of amino acids by activated carbons has been studied by Baur and Wunderly.[20] Evidence of both types of decomposition on adsorption columns of Darco G-60 was obtained. The fraction of "leucine" obtained from a large-scale separation of leucine from glycine weighed nearly as much as the leucine which had been applied to the adsorbent; however, the fraction contained some ammonia and its organic nitrogen content was below the theoretical value for leucine. (The weights had been corrected for inorganic matter washed out of the adsorbent.) Recoveries of phenylalanine and tyrosine from adsorption columns on which these amino acids were separated averaged about 85% for each amino acid, determined colorimetrically. The tyrosine was determined by the Folin and Marenzi method which is not specific for tyrosine but for the phenolic group. If, therefore, only aminolysis had occurred the color test might have been expected to indicate a recovery of tyrosine in the neighborhood of 100%. It is therefore probable that, assuming nearly complete elution, a considerable breakdown of some of the tyrosine molecules occurred.

(19) O. Warburg and E. Negelein, *Biochem. Z.*, **113**, 257 (1921); O. Warburg, *ibid.*, **136**, 266 (1923); E. Negelein, *ibid.*, **142**, 493 (1933); O. Warburg, *Naturwiss.*, **11**, 159 (1923).

(20) E. Baur *Helv. Chim. Acta*, **5**, 825 (1922); *Z. physik. Chem.*, Bodenstein Festband, 162 (1931); K. Wunderly, *ibid.*, **112**, 175 (1924); *Helv. Chim. Acta*, **15**, 721 (1932); **16**, 515, 1009 (1933); **17**, 523 (1934); E. Baur and G. Schindler, *ibid.*, **18**, 1147 (1935); E. Baur and K. Wunderly, *Biochem. Z.*, **262**, 300 (1933); **272**, 1 (1934); additional references may be found in J. W. Hassler, "Active Carbon the Modern Purifier," Industrial Chemical Sales Div., West Virginia Pulp and Paper Company, New York, 1941.

If it should not be possible to prevent decomposition readily, it may nevertheless be of value to develop a chromatographic scheme for separation of all the naturally occurring amino acids. It has been pointed out that the chromatographic method might usefully be applied to small amounts of protein hydrolyzates. This scheme could then be used in conjunction with the isotope dilution method.[21] A sample obtained by the chromatographic method might be contaminated by amino acid decomposition products, as mentioned above, but crystallization should suffice to remove these.

It should be possible also to employ the chromatographic method on a larger scale for the preparation of individual amino acids free from other amino acids.

Several of the separations that have been developed appear to be superior to those available in the literature. The adsorption methods possibly offer advantages in the sharpness of separations and possibly in the saving of time. Compare, for example, the following methods for the separations of leucine and tyrosine, and of glycine and leucine. Habermann and Ehrenfeld[22] separated leucine and tyrosine by refluxing with acetic acid and then treating with 95% alcohol. Leucine remained in solution and the tyrosine was filtered off and recrystallized from water. The leucine filtrate was treated with animal charcoal and the leucine on crystallization contained no trace of tyrosine (most probably because the charcoal adsorbed the last traces of tyrosine). Pfeiffer and Wittka[23] separated leucine and glycine by treating an aqueous solution of these amino acids with ammonium sulfate. In this manner 60% of the leucine was precipitated.

(21) H. H. Ussing, *Nature*, **144**, 977 (1939); D. Rittenberg and G. L. Foster, *J. Biol. Chem.*, **133**, 737 (1940); S. Graff, D. Rittenberg and G. L. Foster, *ibid.*, **133**, 745 (1940).

(22) J. Habermann and R. Ehrenfeld, *Z. physiol. Chem.*, **37**, 18 (1902).

(23) P. Pfeiffer and F. Wittka, *Ber.*, **48**, 1041 (1915).

It seems possible, then, that the method presented in this paper will be useful both for isolation of amino acids and for their preparation. It is hoped to extend the method to other amino acids. It should be possible to make certain predictions as to the separability of amino acids on adsorption columns of Darco G-60 charcoal. For this purpose one would use the adsorption isotherms given by the amino acids with Darco G-60,[24] and would assume that the chromatographic regularities shown here hold for other monoamino monocarboxylic acids.

The use of *dl* mixtures in this work was based on the finding that the adsorption column did not separate such mixtures into the respective optically active isomers.

Note added in proof.—Attention should be called to the following two papers in which adsorption analysis was used to separate amino acids: F. Turba, *Ber.*, **74**, 1829 (1941), from *Chem. Abstr.*, **36**, 5494 (1942); and R. J. Block, *Proc. Soc. Exptl. Biol. Med.*, **51**, 252 (1942).

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Summary

A method has been presented which utilizes chromatographic analysis on adsorption columns of a commercial charcoal for the separation of a mixture of glycine, *dl*-leucine, *dl*-phenylalanine, and *l*-tyrosine.

The advantages and disadvantages of the method have been discussed, as well as its possible application to other mixtures of amino acids.

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(24) V. H. Cheldelin, Dissertation, University of Texas, Austin, 1941; V. H. Cheldelin and R. J. Williams, THIS JOURNAL, **64**, 1513 (1942).