PREPARATIVE ISOLATION OF AMINO ACIDS BY CARRIER DISPLACEMENT CHROMATOGRAPHY ON ION EXCHANGE RESINS*

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Chromatographic separations of amino acids on ion exchange resins may be performed on a preparative scale either by elution (2–4) or by displacement (5–12). Some of the exceptional resolving power demonstrated in analytical separations by elution techniques (13, 14) disappears when larger columns with relatively higher loads are used (2).

Displacement chromatography allows large quantities of solute to be chromatographed on modest sized columns (12), but inherent in this technique is the disadvantage that solutes are mixed at zone boundaries. Furthermore, compounds of very similar structure are apt to emerge with large overlapping zones or are mixed throughout. These mixed fractions must be discarded or rechromatographed, thereby reducing the yield or increasing the labor. Quantitative recovery of pure material is theoretically impossible.

With minor exceptions, bases and acids emerge from cation or anion exchange resin columns in an order inversely related to their strength (15). That this order is maintained, whether or not the substance is amphoteric, is evident from the data of Partridge (8), Partridge and Brimley (12), and Shewan *et al.* (16, 17). Glucosamine, ammonia, and trimethylamine oxide assume predictable positions in the displacement order. Because the two latter compounds, both easily removable free bases, eliminate the overlapping zone between adjacent ampholytes, it seemed likely that other bases might be found which would completely separate other pairs of amino acids in cationic displacement experiments and that acids would probably act in the same capacity with anion exchange resins. These separating acids and bases are called "carriers," the term adopted by Tiselius and Hagdahl (18) to describe a series of alcohols which bring about separation of several amino acids and peptides on charcoal-Super-Cel displacement columns. Although non-ionic carrier displacement has had several applications (19–24) and the possible use of the principle in ion exchange chromatography has been recognized (12, 25), no previous

* A preliminary report of this research has appeared in abstract form (1).

reports of its application seem to have been published. The practicability of separating mixtures of amino acids is illustrated by descriptions of two of the most useful variations of this procedure.

Materials and Methods

Apparatus—Separations were performed with the set of columns described in Table I, the exact dimensions of which are not critical. Each column was fitted with a sintered glass plate.[1] Ground glass joints enabled the columns to be used in cascades and in various combinations. Double sectioned jacketed columns (Fig. 1) had rubber stoppers placed so that a snug fit into the jacket allowed the ground glass joint to seat firmly.

Columns were operated by gravity flow, constant pressure being maintained by a simple fluid-leveling mechanism (Fig. 1). Fractions were collected in 15 × 150 mm. tubes on a time-actuated collector[2] and dried with a rotary evaporator (26). Filtrations were performed on sintered glass microfunnels.[3]

Cooling and heating of jacketed columns were carried out with a circulating water bath[4] modified for refrigeration by the addition of a copper coil attached to a compressor.[5]

Resins—Dowex 50-X4 (200 to 400 mesh) was graded by repeated sedimentation in distilled water. All material which settled in 6 minutes when 200 ml. of resin were suspended in a filled 2 liter graduate was retained. This was passed through cheesecloth and then heated with stirring at 50° in 5 volumes of 2 N NaOH for 5 hours (12). The resin was then poured into a large column, washed with water, and regenerated with 6 N HCl until the effluent was colorless. After being washed with water, the material was stored in the wet state. Used Dowex 50 was collected in a reservoir and, when needed, regenerated in a column with 5 volumes of 6 N HCl.

Dowex 2-X8 (100 to 200 mesh), chloride form, was repeatedly sedimented in distilled water to remove the "fines." Just before use, it was regenerated with 1 N NaOH free from oxygen and CO_2 in a large column. Percolation was continued until the effluent was free from chloride. After being washed to neutrality with boiled and cooled water, the suspended material was

[1] These were ground from coarse sintered plates of discarded glassware. Short sections of glass tubing identical to that of the columns were mounted in the chuck of a drill press. A water paste of 400 mesh alundum was the abrasive.

[2] Spiral fraction collector, No. T-10, Gilson Medical Electronics, Madison, Wisconsin.

[3] Sintered funnel, No. 36060, 2 ml., Corning Glass works, Corning, New York.

[4] Constant temperature circulating bath, No. 4-94, American Instrument Company, Inc., Silver Spring, Maryland.

[5] Compressor, ⅓ h.p., No. A-38F, Brunner Manufacturing Company, Utica, New York.

poured to the mark of the appropriate column (Table I) and regenerated with a 1 M solution of the desired acid. Pressure (about 50 mm. of Hg) was applied to hasten this step. Used resin was converted to the chloride form to be regenerated just before re-use.

TABLE I

Description of Resin Columns

Column No.	Length*	Volume†	Resin	Resin form‡	Footnote references
	cm.	*ml.*			
1	20	10	Dowex 2-X8	Acetate	§
1A	55	30	" 2-X8	Lactate	‖
2	9	6	" 50-X4	Pyridine	§
2A	35	19	" 50-X4	Hydrazine, hydrogen	‖
3	81	50	" 50-X4	Pyrazole, 1-isopropylpyrazole, hydrogen	‖
4	50	30	" 2-X8	Succinimide	‖
5	59	43	" 50-X4	Hydrogen, isopropylpyrazole, hydrogen	§¶
5A	10	3.5	" 50-X4	Hydrogen	‖¶
6	117	90	" 2-X8	Hydroxide, succinimide	‖
7	50	70	" 50-X4	Hydrogen, pyrazole	**††
7A	18	10	" 50-X4	"	‖††
8	81	50	" 50-X4	" nicotinamide	‖

* Length from sintered plate to a mark which designated the top of the resin bed. The tubes were at least 3 to 4 cm. longer at each end in addition to a ⅋ joint. The inner diameters of the tubes were 0.7 to 1.3 cm.

† Volume of settled resin needed to fill column to mark.

‡ Some forms of these resins, *i.e.* acetate, lactate, pyridine, pyrazole, 1-isopropylpyrazole, may be stored in the form used. Others are unstable and must be regenerated before use. Multizoned columns are most conveniently prepared by regeneration *in situ*. See the text.

§ 14/35 ⅋ joints, outer at top, inner at bottom.

‖ 14/35 ⅋ outer joint at top, drip tip at bottom.

¶ Jacketed, double sectioned column (Fig. 1) operated at 0°.

** 24/40 ⅋ outer joint at top, 14/35 ⅋ inner joint at bottom.

†† Jacketed, double sectioned column (Fig. 1) operated at 45°.

Dowex 1-X8 (200 to 400 mesh) was sedimented for 10 minute periods in the manner described for Dowex 50. It was converted to the hydroxide form by prolonged percolation with carbonate-free 1 N NaOH. After the effluent became chloride-free, it was washed with water and stored in a refrigerator until used. Throughout the experiments, Dowex 1 and Dowex 2 were used interchangeably with no apparent difference in results.

Pouring of Columns—Better resolution was obtained when columns were poured in sections with the application of pressure from a regulator

set at 10 pounds per sq. in. Upper resin boundaries were leveled between additions of resin or fluid by manual rotation of the columns held in a vertical position.

Reagents—Analytical grades of the various commercially available acids and bases were used without further purification. Succinimide, though commercially available, was synthesized (27) for expedience. Pyra-

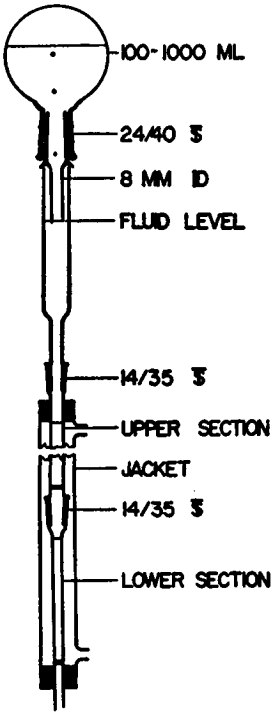

FIG. 1. Jacketed column in sections with fluid-leveling mechanism

zole and several 1-substituted derivatives were prepared by modifications of the procedures of Jones (28).

In the synthesis of pyrazole, equimolar quantities of 1,1,3,3-tetramethoxypropane[6] and hydrazine sulfate were gently refluxed in 60 per cent alcohol (250 ml. per mole) for 2 hours, and the mixture was then distilled to 100°. The residue was diluted with water (200 ml. per mole) and treated with anhydrous Na_2CO_3 until CO_2 evolution ceased. After cooling, the upper phase was separated and the lower phase extracted with two 100 ml. portions of ether. The extracts were combined with the separated upper phase and distilled. The distillate was discarded until solidification occurred in the water-cooled condenser, which was

[6] Obtained from Kay-Fries Chemicals, Inc., New York, New York.

then quickly drained of cooling water. Pyrazole (b.p. 184°) was collected until yellow fumes appeared in the condenser and, when crystallized from 3 volumes of ether, gave a yield of 80 per cent (m.p. 70°). 1-Methylpyrazole was prepared by substituting methylhydrazine sulfate.

Ethyl, isopropyl, *n*-propyl, isobutyl, *n*-butyl, β-hydroxyethyl, and allyl derivatives were prepared by a modification of the method described for 1-benzylpyrazole (28). Sodium shavings (28 gm.) were quickly washed with alcohol and added to 1 liter of cooled absolute alcohol. When the reaction was complete, pyrazole (68 gm.) was added and the mixture gently refluxed for 2 hours during the dropwise addition of 1.5 moles of the appropriate alkyl iodide or bromide. Refluxing was continued until the mixture became neutral. For screening tests, these mixtures, after filtration, were passed into 500 ml. columns of Dowex 50-X8 (200 to 400 mesh), washed with water, and displaced over a collector with 2 M NH_4OH. Fractions were pooled between the visible breakthrough of the pyrazole derivative (also detectable by odor) and the first appearance of a precipitate with ammoniacal $AgNO_3$ (unchanged pyrazole).

In later preparations of the two most useful of these compounds, 1-ethylpyrazole (b.p. 139°) and 1-isopropylpyrazole (b.p. 143°), the reaction mixture was rapidly distilled from the salt and then fractionally distilled to give yields of about 60 per cent (ethyl) and 30 per cent (isopropyl). When the silver salt of pyrazole was treated with alkyl bromides, yields were lower and inconsistent.

The amino acids were obtained from two sources (Table VII) and were of the optical configurations shown in Tables IV and VII.

Spot Tests and Paper Chromatograms—After the techniques had been established, progress of most of the fractionations was followed by spot tests. Half-inch squares were ruled and numbered in pencil on Whatman No. 1 filter paper. Aliquots (approximately 2 μl.) were then spotted with small stirring rods or capillary micropipettes and these were sprayed with a 1 per cent solution of ninhydrin in butanol and heated.

In cases in which amino acids were not widely separated by ninhydrin-negative carriers or in which carriers interfered with ninhydrin color development, one-dimensional paper chromatography was performed on Whatman No. 3 paper in *n*-butanol-acetic acid-water (4:1:1) solvent. Spots (2 μl.) were pipetted from serial fractions at half-inch intervals along a line 3 inches from the edge of a half sheet of paper. Development (descending) for 5 hours gave sufficient resolution for clear identification of all amino acids except the isomeric leucines.

Separation of First Mixture

1 mmole each of fifteen amino acids (see Table IV) plus 0.1 mmole each of cystine and tyrosine were dissolved in 70 ml. of water. The

three basic amino acids were added as the monohydrochlorides. Cystine and tyrosine were dissolved by adding 1 ml. of 1 M NH_4OH and heating before the other amino acids were added.

Primary Separation—The amino acids were first divided into acidic, basic, and neutral groups by passing the mixture successively through Columns 1, 2, and 3 (Table I). Ground glass joints were lightly lubricated on the upper half with a hydrocarbon stopcock grease (not silicone).

Columns 1 and 2 were prepared by pouring resin stored in the appropriate form. Column 3 was prepared by pouring the column in the hydrogen form and then successively adding 75 ml. of 0.2 M 1-isopropylpyrazole and 75 ml. of 0.2 M pyrazole. A funnel of the type shown in Fig. 1 was used for pouring the columns and for adding solutions to them.

After all but 2 to 3 ml. of the amino acid mixture had passed into the top surface of resin, 100 ml. of distilled water were added in portions. Ninhydrin spot tests of the effluent from each of the three columns became negative before all of the water had washed through, and the 170 ml. of effluent were discarded.

Column 1 retained glutamic and aspartic acids and the chloride from the basic amino acid salts. Column 2 retained only the three basic amino acids. Column 3 retained the neutral amino acids and the pyridine displaced from Column 2 by the basic amino acids. The three columns were developed separately but simultaneously.

Separation and Purification of Acidic Amino Acids—Column 1 was detached from the cascade and placed above Column 1A (Table I) which had been filled with resin stored in the lactate form. The pair of columns was developed with 0.2 M citric acid. Fractions containing glutamic acid and those containing aspartic acid were separately pooled (Table II) and taken to dryness with the evaporator. Each was brought into solution on a steam bath with 3 ml. of hot water and crystallized by adding 20 ml. of boiling absolute alcohol.

Separation and Purification of Basic Amino Acids—Column 2 was placed above Column 2A (Table I), which was prepared by pouring the column with resin in the hydrogen form and then adding 50 ml. of 0.2 M hydrazine.[7] The pair of columns was developed first with 50 ml. of 0.2 M NH_4OH[8] and then with 0.2 M piperidine. After the addition of the piperidine, the column assembly was placed over the collector. Because hydrazine masks the ninhydrin spot test and piperidine produces the reaction, a paper chromatogram was performed on each fraction to identify those

[7] Collidine (1) is a less satisfactory carrier for separating histidine and lysine because, when used, the column must be heated to about 50°.

[8] When the carrier NH_4OH was added *before* the amino acids, there was considerable trailing of lysine.

containing each basic amino acid. After the solvent front had run only 2 inches past the origin, accurate identification of fractions was possible.

Fractions containing histidine, lysine, and arginine (Table II) were separately pooled and taken to dryness. The flasks were then attached to a high vacuum manifold and evacuated for 2 to 3 hours to remove the

TABLE II

Development of Columns in Primary Separation of First Mixture

Column	Fraction Nos.*	Volume	Content
		ml.	
1-1A		50	Acetic acid†
	1–5	61	Glutamic acid
Dowex 2	6–10	60	Lactic acid†
	11–13	36	Aspartic acid
			Citric acid†
2-2A		180	Water, pyridine†
	1–3	23	Histidine
Dowex 50	4–5	15	Hydrazine†
	6–9	29	Lysine
	10–13	29	Ammonia†
	14–17	29	Arginine
			Piperidine
3		192	Water†
	1–6	45	Group I: serine, threonine, proline
Dowex 50	7–9	22	1-Isopropylpyrazole†
	10–23	104	Group II: "aliphatic" and sulfur-containing amino acids
	24–27	30	Pyrazole, cystine (trace)
	28–37	74	Group III: phenylalanine, tyrosine
			Pyridine†

* Collector set to change every 20 minutes.

† Ninhydrin spot test negative for amino acids.

last traces of free base. After addition of 2 ml. of 1 M HCl, the material was again taken to dryness and redissolved in a minimum of water. Alcohol (10 ml.) was first added and then pyridine (0.5 ml.) to crystallize the monohydrochloride salts (2).

Separation of Neutral Amino Acids into Groups—Column 3 was developed with 0.2 M pyridine solution. Spot tests revealed a clear separation of the amino acids into three groups. Paper chromatograms identified the first group as proline, serine, and threonine, the second group as glycine, alanine, valine, methionine, cystine, leucine, and isoleucine, and

the third group as phenylalanine and tyrosine (Table II). Because of its low solubility, cystine had crystallized from several fractions and had trailed across the break between Groups II and III. All fractions with precipitated cystine were filtered through a sintered glass microfunnel before they were pooled. This was set aside for the later collection of more cystine.

The groups were pooled and Groups I and III taken to dryness. Phenylalanine and tyrosine were not further separated in this experiment and were crystallized together.

Separation of Proline—The mixture of proline, serine, and threonine was taken up in 20 ml. of water and passed through Column 4. Because succinimide slowly hydrolyzes to succinamic acid under alkaline conditions, this column was regenerated from the washed hydroxide resin just before use. The column was placed over the collector before the amino acids were added. Spot tests revealed the fractions containing proline and those containing the threonine-serine mixture (Table III).

The pooled proline fractions were passed through a 2 ml. column of Dowex 50 in the hydrogen form, washed with water, and displaced into an evaporating flask with 10 ml. of 1 M ammonium hydroxide. After being taken to dryness, the residue was dissolved in 3 ml. of hot absolute alcohol and precipitated with 10 ml. of ether.

Separation of Serine and Threonine—The pooled mixture of serine and threonine was next passed into Column 5 (Table I). This was prepared by pouring 40 ml. of Dowex 50-hydrogen into the column and then adding 4 ml. of 1-ethylpyrazole in 100 ml. of water. After this solution had been washed into the resin, 3 ml. of hydrogen resin were added and then the amino acid mixture was transferred to the column. When the solution had entered the resin, the column was fitted into the jacket and coupled to Column 5A, the lower section. The column was cooled to 0° by means of a circulating water bath[4] containing 10 per cent ethanol and developed with 0.1 M pyridine.

Because of the considerable lapse of time before the emergence of threonine, effluent was first collected in a single vessel. Collection of fractions was started after the visible advancing boundary of the carrier had entered Column 5A. Paper chromatography was performed on each fraction which gave a positive spot test. Fractions containing threonine and serine (Table III) were separately pooled and taken to dryness. Each residue was redissolved in 1 ml. of water on a steam bath and the amino acids were crystallized by the addition of 15 ml. of boiling alcohol.

Separation of Alanine, Methionine, and Cystine from Group II of Neutral Amino Acids—Column 6 was prepared by pouring 80 ml. of freshly re-

generated and washed Dowex 2-hydroxide and then converting this with 10 gm. of succinimide dissolved in 100 ml. of degassed water. After this solution had been forced through the resin, 10 ml. of Dowex 2-hydroxide

TABLE III

Development of Columns Separating Neutral Amino Acids of First Mixture

Column	Fraction Nos.	Volume	Content
		ml.	
4*	1	12	Succinimide†
	2–6	61	Proline
Dowex 2	7–10	54	Succinimide†
	11–16	81	Serine, threonine
			Succinimide†
5-5A‡		375	Water, 1-ethylpyrazole†
	1–9	42	Threonine
Dowex 50	10–33	112	Serine
6*		169	Succinimide†
	1–4	44	Alanine
Dowex 2	5–15	117	" glycine, valine, "leucines"
	16–19	42	"Leucines"
	20–33	141	Succinimide†
	34–42	90	Methionine
	43–57	150	Cystine
			Acetic acid
7-7A‡		336	Water, pyrazole†
	1–4	43	Glycine
Dowex 50	5–7	32	" alanine
	8–9	21	Alanine
	10–11	21	Pyrazole†
	12–21	101	Valine
	22–25	30	Pyrazole†
	26–32	65	Alloisoleucine
	33–38	55	Leucine, isoleucine
			Pyridine

* Collector set to change every 10 minutes.
† Ninhydrin spot tests negative for amino acids.
‡ Collector set to change every 20 minutes.

were added to the top of the column and the pooled mixture of "aliphatic" and sulfur-containing amino acids from Column 3 was allowed to flow through by gravity. After the mixture had been rinsed into the resin, the column was developed with 0.2 M acetic acid while 10 minute fractions were collected.

Paper chromatograms (Table III) revealed that most of the alanine emerged unmixed. Methionine and cystine were well separated from the mixture and, except for a slight overlap, from each other. Unmixed fractions of each of these were pooled and passed through separate 2 ml. columns of Dowex 50-hydrogen. After washing with water, they were displaced with 10 ml. of 1 M NH_4OH. Methionine was taken to dryness, dissolved in 1 ml. of water on a steam bath, and crystallized by the addition of 15 ml. of absolute alcohol. Cystine was taken to dryness, suspended in 3 ml. of water, and filtered through the sintered microfunnel used to collect the cystine previously precipitated. The displaced alanine solution was set aside for the later addition of the remaining alanine.

Separation of Glycine, Alanine, Valine, and "Leucines"—All mixed fractions of these amino acids from the previous separation were pooled and passed into Column 7-7A. The upper section, prepared mainly from resin in the pyrazole form, had 4 ml. of hydrogen resin at the top. The column was maintained at 45° during its development with 0.2 M pyridine.

The remaining alanine (approximately 10 per cent) emerged still mixed with glycine (Table III). Valine was well separated, and, although separation of isoleucine and leucine seemed to have taken place, this proved to be merely a separation of alloisoleucine present as a contaminant in the isoleucine (see below).

Unmixed fractions were pooled and taken to dryness. The fractions of alanine and glycine which were still mixed were separated on a sheet of heavy filter paper[9] with *n*-butanol-acetic acid-water (4:1:1) solvent.

Recoveries—Table IV lists the per cent yield of each amino acid added to the mixture. The high recovery of valine was the result of an error in weighing the original material. The isolated amino acid was chromatographically pure. A previous experiment had given the result in parentheses. The yield of phenylalanine and tyrosine is that of the mixture.

All of the amino acids but glycine and alanine appeared as white powders or crystals. These two had acquired a yellow contaminant from the filter paper. Recrystallization in most cases did not greatly reduce the yield, as seen in the last column of Table IV. An accident in a vacuum oven explains the missing values in this column.

Separation of Second Mixture

A second mixture was prepared to simulate the content of amino acids in 2 gm. of bovine serum albumin (29). Because of the paucity of methionine and tryptophan in this protein, these were each increased in amount (Table VII).

[9] No. 470A, Carl Schleicher and Schuell Company, Keene, New Hampshire.

The mixture was brought into solution with an equimolar quantity of hydrochloric acid and then to a volume of 30 ml. After addition of the equivalent amount of solid pyrazole, a crop of crystals formed overnight which, when filtered, represented 75 per cent of the cystine and no tyrosine. Addition of the same molar quantity of ammonia and overnight cooling produced a second crop of crystals which were mainly tyrosine with some cystine. These also were removed by filtration on a sintered microfunnel. The filtrate was taken to dryness to remove ammonia

TABLE IV

Recovery of Amino Acids from First Mixture

Amino acid	First crystallization	Recrystallization
	per cent	*per cent*
L-Glutamic acid	96.0	92.4
DL-Aspartic acid	88.2	
L-Histidine	97.8	94.3
L-Lysine	80.0	71.2
L-Arginine	89.6	
DL-Threonine	88.0	84.0
DL-Serine	84.4	80.4
DL-Methionine	79.0	75.3
L-Proline	95.8	93.2
"Leucines"*	90.3	
DL-Valine	138.3 (84.9)†	
DL-Alanine	99.0	77.1
Glycine	65.4	57.3
DL-Phenylalanine L-Tyrosine‡	87.2	
L-Cystine‡	50.8	

* DL-Leucine (1.0 mmole), L-isoleucine (0.5 mmole), D-alloisoleucine (0.5 mmole).

† Previous experiment.

‡ 0.1 mmole added.

and the amino acids and pyrazole were redissolved in 50 ml. of water. The subsequent procedure is summarized by a flow diagram in Fig. 2.

Primary Separation—This separation was performed on a single column (117 cm., 90 ml.) of Dowex 50. 15 mmoles of each of the first three carriers, 1-isopropylpyrazole, pyrazole, and nicotinamide, in 0.2 M solution were first added in turn to the column.

After the amino acid mixture had been put on the resin, 15 mmoles of each of the remaining carriers, pyridine, hydrazine, and ammonia, were added in 0.2 M solution. The final displacing agent, piperidine, was added in 0.1 M solution.

The amino acids emerged in groups and singly as shown in Table V.

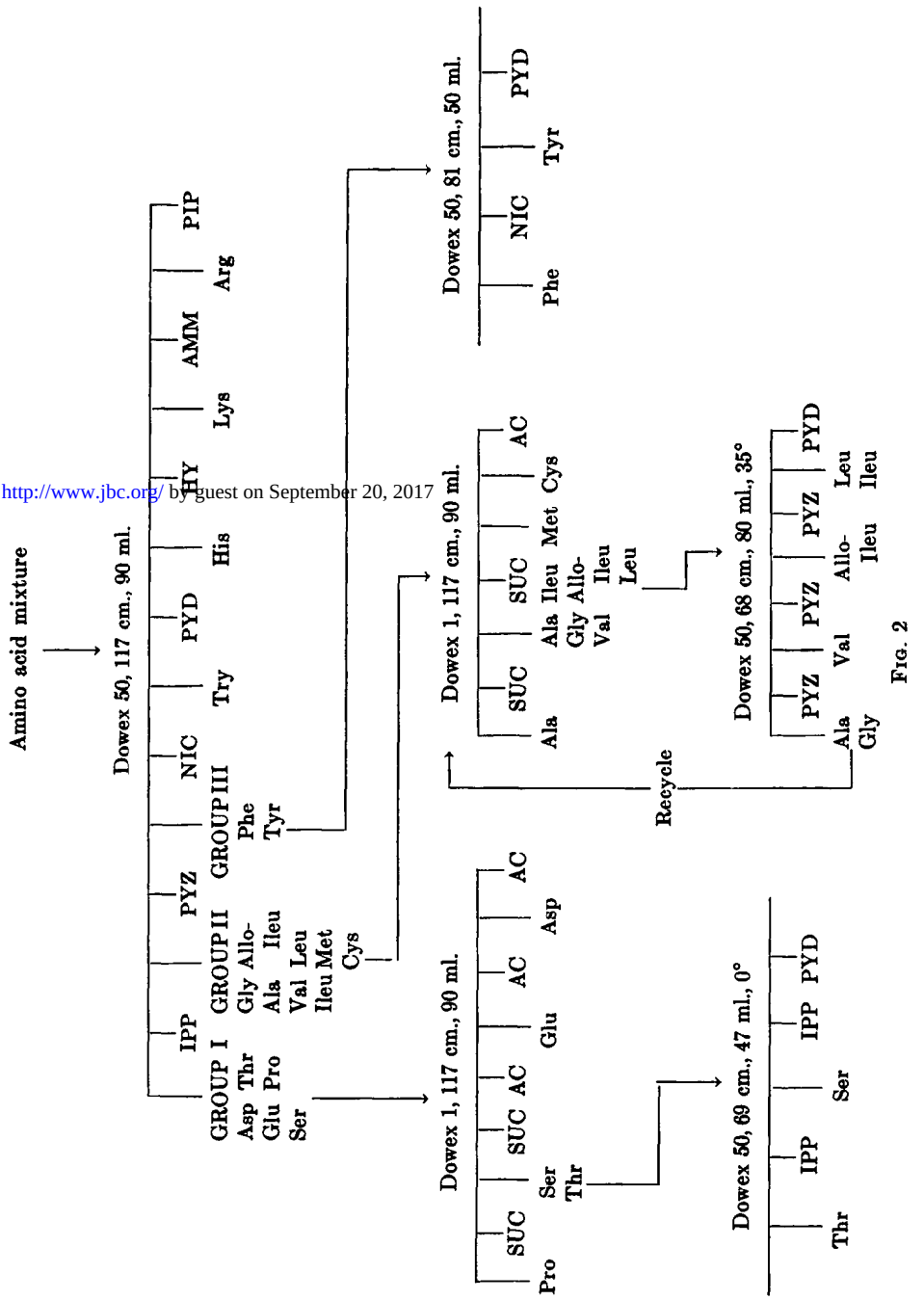

FIG. 2

Group I contained glutamic and aspartic acids, as well as the three amino acids present in the first experiment. Groups II and III were the same as described previously.

Separation of Group I—Column 6 (Table I) was next poured with Dowex 1 in the succinimide form and the pooled fractions of proline, threonine, serine, glutamic acid, and aspartic acid at a volume of 20 ml. were then

TABLE V

Development of Column in Primary Separation of Second Mixture*

Fraction Nos.†	Volume	Content
	ml.	
	400	Water
1–10	75	Group I: glutamic acid, aspartic acid, proline, threonine, serine
11–15	38	1-Isopropylpyrazole‡
16–47	270	Group II: glycine, alanine, valine, "leucines," methionine, cystine
48–70	167	Pyrazole‡
71–82	90	Group III: phenylalanine, tyrosine
83–89	53	Nicotinamide‡
90–113	175	Tryptophan
114–118	37	Histidine
119–127	68	Hydrazine‡
128–138	77	Lysine
139–157	128	Ammonia‡
158–164	50	Arginine
		Piperidine

* Dowex 50-X4, 117 cm., 90 ml.

† Collected each 20 minutes after the visible boundary reached the lower end of the column.

‡ Ninhydrin spot tests negative for amino acids.

added. After these had been washed in, the column was developed with 0.2 M acetic acid (Table VI). The amino acids emerged in the order given, proline and the acidic amino acids being well separated. Serine and

FIG. 2. Separation of second mixture. Carriers and their contractions are as follows: IPP, 1-isopropylpyrazole; PYZ, pyrazole; NIC, nicotinamide; PYD, pyridine; HY, hydrazine; AMM, ammonia; PIP, piperidine; SUC, succinimide; AC, acetic acid. Amino acids and their contractions are as follows: Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Thr, threonine; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Ileu, isoleucine; Allo-Ileu, alloisoleucine; Leu, leucine; Met, methionine; Cys, cystine; Phe, phenylalanine; Tyr, tyrosine; Try, tryptophan; His, histidine; Lys, lysine; Arg, arginine.

TABLE VI

Development of Columns Separating Amino Acids in Groups I to III of Second Mixture

Column	Fraction Nos.*	Volume	Content
		ml.	
6†	1–2	40	Succinimide‡
	3–5	69	Proline
Dowex 1	6–11	117	Succinimide‡
	12–17	122	Serine, threonine, methionine sulfoxide
	18–19	45	Succinimide, acetic acid‡
	20–24	83	Glutamic acid
	25–32	167	Acetic acid‡
	33–128	120	Aspartic acid
			Acetic acid‡
5-5A		325	Water, 1-isopropylpyrazole
	1–8	33	Methionine sulfoxide
Dowex 50	9–10	8	1-Isopropylpyrazole
	11–50	165	Threonine
	51–75	104	Serine
			1-Isopropylpyrazole
6†	1–12	90	Succinimide‡
	13–20	55	Alanine
Dowex 1	21–50	176	" glycine, valine, "leucines"
	51–61	55	Succinimide, leucine (trace)
	62–81	92	Methionine
	82–83	8	" cystine
	84–115	150	Cystine
7-7A		350	Water, pyrazole‡
	1–2	23	Glycine
Dowex 50	3–5	34	Alanine, glycine
	6–7	22	"
	8	11	Pyrazole‡
	9–17	89	Valine
	18–20	27	Pyrazole‡
	21–30	74	Alloisoleucine
	31–33	16	Pyrazole‡
	34–56	120	"Leucines"
			Pyridine‡
8		155	Nicotinamide‡
	1–12	110	Phenylalanine
Dowex 50	13–15	27	Nicotinamide‡
	16–30	135	Tyrosine
			Pyridine‡

* Collected every 20 minutes.

† Dowex 1-succinimide.

‡ Ninhydrin spot test negative.

threonine were mixed with methionine sulfoxide. These three were separated on the refrigerated Column 5 as described for the first mixture except that 1-isopropylpyrazole (4 ml.) was used as the carrier. Methionine sulfoxide came off ahead of threonine and was well separated.

TABLE VII

Recovery of Amino Acids from Second Mixture

Amino acid	Initial amount	First crystallization	Recrystallization
	mg.	*per cent*	*per cent*
L-Glutamic acid*	339.2	96.0	94.2
DL-Aspartic acid	221.7	88.0	86.4
L-Histidine·HCl·H_2O	102.3	93.6	89.0
L-Lysine·HCl	319.0	95.6	90.6
L-Arginine·HCl*	151.6	88.9	84.5
DL-Threonine	131.6	88.1	84.0
DL-Serine	90.5	82.8	83.6
DL-Methionine*	150.1	69.9†	66.4†
L-Cystine*	132.0	97.1	83.8
L-Proline	121.3	83.5	73.5
DL-Leucine	248.8	88.9	80.5
L-Isoleucine	27.2		
DL-Alloisoleucine‡	27.2	90.5	80.4
DL-Valine*	128.9	98.4	93.5
DL-Alanine*	101.0	82.4	69.2
Glycine	40.9	54.8§	54.0§
DL-Phenylalanine	137.1	93.0	88.5
L-Tyrosine	105.3	91.6	82.8
DL-Tryptophan*	104.9	94.2	85.4

* These chemicals from Distillation Products Industries Division of Eastman Kodak Company; all others from Nutritional Biochemicals Corporation.

† Corrected for 4.8 per cent yield of methionine sulfoxide.

‡ See footnote 10 of the text.

§ Some material lost on fraction collector.

Separation of Group II—This separation was performed as for the first mixture except that Dowex 1 replaced Dowex 2 in Column 6, and the mixed fractions of alanine and glycine were separated by being rechromatographed on Column 6 rather than on paper.

Separation of Group III—Phenylalanine and tyrosine were separated on Column 8 (Table I), Dowex 50 in the nicotinamide form. When pyridine was used to displace, there was excellent separation (Table VI).

Separation of Cystine and Tyrosine—The mixture of these amino acids which had crystallized upon addition of ammonia was redissolved by adding an equivalent amount of hydrochloric acid and then was placed on a

column of Dowex 50 in the pyrazole form (30 ml., 50 cm.). When developed with 500 ml. of pyridine, cystine preceded tyrosine in very dilute solution.

Recoveries—Table VII gives the yield of each of the amino acids in the second mixture. The average recovery was 87.4 per cent upon the first crystallization and 81.6 per cent upon recrystallization. Glycine gave the lowest yield as a result of loss of some effluent. Methionine also gave a low yield and this was partly the result of oxidation of the amino acid. Nearly 5 per cent of the added methionine was recovered as methionine sulfoxide in Group I (Table VI).

Purity—Heavily spotted paper chromatograms of all amino acids showed traces of contamination only in those that had displayed slight overlapping zones. Glycine contained a trace of alanine. Serine and threonine each had faintly detectable amounts of the other. Methionine contained a trace of leucine as well as cystine and methionine sulfoxide. Tyrosine still showed a slight contamination with cystine which disappeared upon recrystallization.

Separation of Isoleucine and Alloisoleucine—For a considerable time during the course of this work, it appeared that isoleucine and leucine were separable on the heated Dowex 50-pyrazole column. However, consistent low yields of "isoleucine" and greater than theoretical yields of "leucine" pointed to an impurity in the isoleucine. The development of a column to which the only amino acid added was 1 mmole of "DL-isoleucine"[10] gave two completely separated zones of equal size. When 50 mg. of this mixture were added to 250 mg. of DL-leucine, the second zone was the larger. When 10 mg. of the "DL-isoleucine" were added to 35 mg. of L-isoleucine,[11] the second zone was again the larger. The first zone is, therefore, alloisoleucine.

DISCUSSION

Although acidic or basic strength is of highest importance in the determination of the displacement order of a group of substances, other factors such as aromaticity and molecular size also contribute (15). To separate two amino acids, a search is made for carrier compounds of intermediate or at least similar pK values. One also considers solubility, reactivity with ninhydrin, ease of removal, and cost. The final selection must, of course, be based upon experiment.

With amino acids which have dissociation constants and resin affinities

[10] The "DL-isoleucine" was in reality 50 per cent L-isoleucine and 50 per cent D-alloisoleucine (personal communication, Nutritional Biochemicals Corporation, Cleveland, Ohio).

[11] Kindly donated by Dr. Joseph Fruton.

almost identical to one another, it is probably impossible to find substances which function as ideal carriers. One may cite the fact that there is little tendency for certain groups of three amino acids to separate at all by ordinary displacement chromatography (12). Obviously one of the three amino acids should have an intermediate affinity for the resin, but, despite this, it does not function as a carrier to separate the other two.

It has been found that an acid or base which functions as a carrier to separate two groups of amino acids may often be useful in the separation of the amino acids of either group by elution. The resin is first saturated with the acid or base and, after the amino acid mixture is added, the column is developed with a dilute solution of the same acid or base. Pyrazole, nicotinamide, 1-isopropylpyrazole, succinimide, and acetic acid are used in this way.

Empirically, the capacity of columns in these techniques is much greater than with elution chromatography. Consequently, columns can be much smaller and volumes of effluent are much less. The first separation was performed with only 200 ml. of Dowex 50 and 160 ml. of Dowex 2 resin compared with about 14 liters of resin used to separate a similar mixture by an elution method (2). The total volume of effluent in the first separation described here was 3200 ml., while in the elution method the volume was approximately 65 liters. Isolation of the amino acids from the effluent is also much simpler with carrier displacement because the carriers are usually volatile or are soluble in alcohol. When neither is the case, they may be removed by passing the pooled effluent through a small column of resin chosen to retain the amino acid but not the carrier. Desalting is never necessary. Because of the small resin volumes, the effluent contains less material extracted from the resin than with elution, and less care need be taken in order to obtain colorless products.

The chief disadvantage of this method is that isoleucine and leucine have not as yet been separated. Furthermore, alanine and glycine are completely resolved only by recycling. The necessity for several columns, though a disadvantage, actually expedites the separation because two or more columns may be developed simultaneously.

Effects of Temperature—Partridge and Brimley (10) ascribe the improvement in separating certain amino acids on heated columns to the partial elimination of the effect of van der Waals forces. By the present technique serine and threonine do not separate very well at 25°, separate partially at 10°, and separate very well at 0°. This demonstrates that a second mechanism is probably operative. In the refrigerated separation, threonine emerges first. If increased van der Waals forces at the lower temperature promoted the separation, one would expect that the larger molecule, threonine, would be the one retarded. It is likely that here the alteration

in temperature effects a separation by differentially changing the dissociation constants of serine and threonine.

Variations in Technique and Possible Improvements—The carrier displacement technique has many possible variations and the one chosen will depend upon which amino acids are to be isolated. Two variations are described here. The first has advantages if only a single or a few amino acids are to be separated. The second is preferable when the objective is the isolation of the eighteen most common amino acids of protein hydrolysates. Numerous improvements in the method will undoubtedly be made in time. The discovery of additional carriers should improve resolution. The failure of pyridine to give a complete separation of tryptophan from histidine suggests that a compound slightly more basic would improve the method. Hydrazine is not an ideal carrier because of its slight instability and preliminary tests indicate that tris(hydroxymethyl)-aminomethane separates histidine and lysine and may thus replace hydrazine. Creatine separates phenylalanine from β-alanine, and nicotinic acid (1) functions like pyrazole, separating leucine from phenylalanine. Being an amphoteric substance, nicotinic acid is more difficult to remove than pyrazole and its use has been abandoned.

In addition to the finding of new carriers, three other conditions may be varied with the possibility of improving separations. Temperature may be raised or lowered, as already discussed. Elevating the temperature may make the carrier run ahead of its normal sequence, as is seen with pyrazole or 1-isopropylpyrazole, or it may have the reverse effect as it does with collidine (1). The brand or type of resin has some effect on the displacement order, as illustrated by the difference between the position of proline on a sulfonated phenolic resin *versus* that on a sulfonated polystyrene resin (12). Finally, the addition of alcohol or other less polar solvents will alter the relative affinities of solutes for the resin (12). Despite several attempts, no very useful applications of these latter two devices have been discovered.

It seems reasonable that for analytical work the solutions could be reduced in proportion to the quantity of amino acid. If so, one could separate 1 mg. of each amino acid with approximately 4 ml. of resin and 32 ml. of developing solutions.

SUMMARY

A new procedure for separating all but two of the common amino acids by displacement chromatography has been devised. It involves the use of bases and acids that have sorption affinities intermediate between those of the amino acids. These "carrier" substances assume positions in the displacement order between the amino acids being separated, and many of

the mixed zones that normally occur are eliminated. In some cases, the compounds used as carriers serve also to separate closely related amino acids by elution analysis. The method is capable of effecting the separation of at least a millimole of each amino acid on a series of columns containing a total of only 360 ml. of resin. Volumes of effluent are correspondingly small and desalting is unnecessary.

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