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Separation of Diastereoisomers by Chromatographic Fractionation; the Resolution of Optical Isomers*

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Summary

Baker and Williams's chromatographic fractionation technique, employing simultaneous temperature and solvent gradients along a column, has been successful in separating three diastereoisomer salt derivatives derived from optical isomers. The *d*- and *l*-α-phenylethylamine diastereoisomers of *d*-mandelic acid and the *d*- and *l*-tartrates of *l*-brucine were separated by approximately 60%. A partial separation was achieved on the *d*- and *l*-histidine salts of *d*-tartaric acid. An aqueous–alcohol–acetonitrile solvent system was used for the separations. Irregular diastereoisomer formation and dissociation of diastereoisomers were problems discussed in the development of the analytical method.

There is no generally applied method for the resolution of a given pair of enantiomers. Considerable research effort has been spent trying to resolve enantiomers directly, that is, without prior chemical transformation to corresponding diastereoisomers. This effort has been partially successful using paper and ion-exchange chromatography with optically active adsorbants (*1,2*). Also, gas-liquid chromatography has been successfully applied to this problem using optically active liquid stationary phases (*3*). Many of these methods are relatively rapid and require little handling. However, certain factors limit

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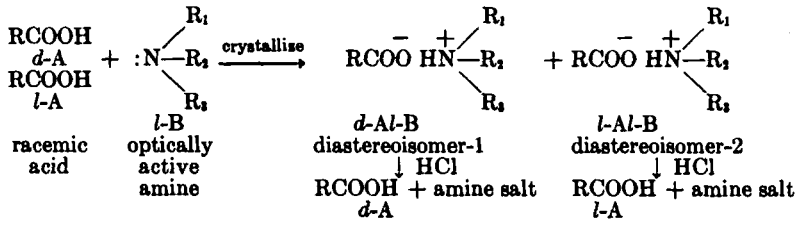
the practical usefulness of these interesting separation methods. The degree of resolution is usually less than 20% and the sample sizes are small.

More complete resolutions have resulted from changing optical isomers to diastereoisomers which are then separated by virtue of their different physical properties. The separation methods used here include fractional crystallization, distillation, and adsorption chromatography (*4–6*). After separating the diastereoisomers, the respective optical isomers are then regenerated. Although these techniques usually require more experimental time than direct methods, the degree of separation is generally between 50 and nearly 100%.

The oldest, and most frequently used resolution technique involves the fractional crystallization of diastereoisomers. In this approach, the racemate is allowed to react with an optically active compound forming two diastereoisomers. The reactions usually involve simple salt formation of optically active bases (B) and acids (A):



The diastereoisomers studied here have been separated using fractional crystallization techniques. Ingersoll and co-workers (*7*) resolved α-phenylethylamine using salts of *l*-bromocamphor sulfonate and mandelic acid. A variety of other resolving agents has also been used to separate the racemic amine. Ingersoll and Little (*8*) used 6,6′-dinitrodiphenic acid as the resolving agent, whereas Helferich and Pontz (*9*) resolved the amine via the insoluble complex formed by the (+) antipode with 2,3,4,6-tetraacetyl-D-glucose. Natural *d*-tartaric acid was used exclusively as the resolving agent in two papers (*10,11*) and in conjunction with *l*-malic acid in another investigation (*12*).

Pasteur (*13*) prepared the brucine salts of *d*- and *l*-tartaric acid, whereas Ladenburg and Fischl (*14*) separated the salts by fractional crystallization in neutral solutions. McKenzie and co-workers (*15*) resolved tartaric acid by virtue of its complex formation with *l*-malic acid. Other more common resolving agents used to resolve tartaric acid by fractional crystallization are *d*-quinicine (*16*) and *d*-N-methylamphetamine (*17*).

Pyman (*18*) was able to resolve racemic histidine, without prior acetylation, using *d*-tartaric acid as the resolving agent. Finally, Dakin (*19*) has reported the resolution of malic acid by fractional crystallization using *d*-cinchonine as the resolving agent.

Baker and Williams, (*20*) chromatographic fractionation technique was introduced in 1956 and has been successfully applied to the fractionation of a large number of industrial polymers. A fractionation was usually initiated in the following way. The polymer samples were coated on a small amount of 0.1-mm glass beads by evaporation from a good solvent. The beads were then added to the top of the glass-bead packing in the column as a slurry in poor solvent. After a stabilized temperature gradient was imposed down the length of the column (usually from approximately 60°C at the top to 10°C at the base of the column), the column was eluted with good solvent, the composition of which increased exponentially.

Baker and Williams described the separation mechanism as a continuous process. Some of the polymer dissolved in the warm top portion of the column upon contact with an increasing amount of good solvent. The polymer was eluted as a saturated solution to a cooler region of the column where it precipitated. Dissolution of the polymer occurred again as a higher concentration of good solvent became present. This process continued throughout the length of the column until the polymer emerged as a saturated solution at the base of the column. The polymers were assumed to have a positive temperature coefficient of solubility.

Baker and Williams's fractionation technique has been used in application outside the polymer field. Schulz and Purdy (*21*) reported the use of the column method in the separation of several stereoisomeric hormone mixtures. Schelz (*22*) extended the application to the study of isomeric steroid systems. A mixture of the sterols, ergosterol and lumisterol, was separated. Also, Van den Tempel and co-workers (*23*) reported the successful fractionation of three impure glycerides.

EXPERIMENTAL

Chromatographic Fractionation Apparatus

The Pyrex glass column (Fig. 1) was filled with a slurry of micro (0.1-mm) borosilicate glass beads (Microbeads, Inc., Jackson, Miss.) in acetonitrile (J. T. Baker Chemical Co., Phillipsburg, N.J.) Aluminum foil was wrapped around the column several times to provide an

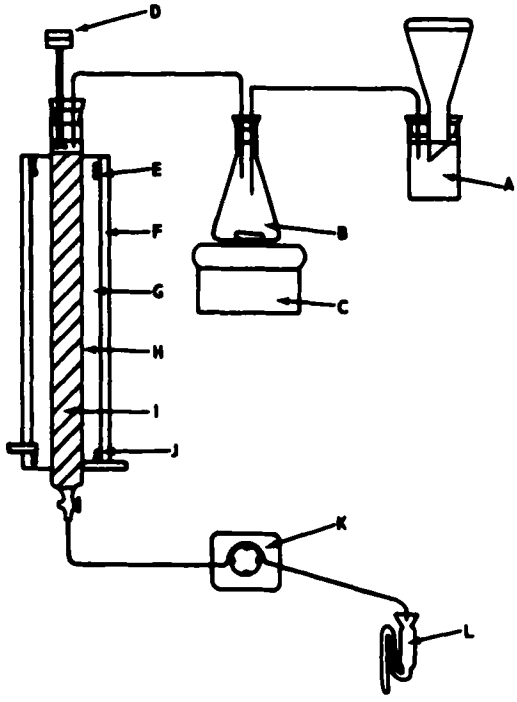


FIG. 1. Chromatographic fractionation apparatus. (A) Good solvent reservoir; (B) mixing vessel; (C) magnetic stirrer; (D) electric stirrer; (E) heating coil; (F) insulation; (G) aluminum jacket; (H) column tube; (I) column packing; (J) cooling coil; (K) peristaltic pump; (L) siphon.

intimate contact with a surrounding aluminum jacket. The jacket served to impose a linear temperature gradient down the length of the column.

The top of the aluminum jacket was heated with four turns of #26 Chromel A wire. This heating wire was attached to a Bakelite terminal block at the head of the column and electrically controlled by a Variac in line with a constant voltage regulator.

The base of the aluminum jacket was cooled by water circulated through copper tubing which was wound in grooves cut into the jacket. Silicon grease served to provide the best thermal contact between the copper tubing and the jacket. Rubber tubing connected the copper tubing to a circulating pump which circulated water from a cooled water reservoir. The reservoir contained some antifreeze and was

maintained at 0° ± 2°C by a thermostated cooling compressor (Ranco, Inc., Columbus, Ohio). The entire aluminum jacket was insulated by several alternating layers of asbestos tape and glass fiber.

The solvent concentration gradient was provided by the constant-volume mixing vessel. This vessel was a 250- or 500-ml volumetric flask shortened at the neck, and it contained a magnetic stirrer. The good solvent reservoir was the combination of a 150-ml beaker and inverted volumetric flask. The neck of the volumetric flask was cut at an angle for best exchange of air and solvent. The feed system was constructed so that the solvent level in the column was constant and dependent on the level of good solvent in the 150-ml beaker. The inverted reservoir replenished the supply of solvent in the beaker and maintained the solvent level. The beaker, mixing vessel, and column were open to the atmosphere. The small motor above the column stirred the solvent at 1 rps. The solvent feed system was reliable and automatic and required no attention over the period of a fractionation. All solvents were degassed before a run by boiling for a brief period of time.

The fractionation flow rates were controlled with a perastaltic-action variable speed pump (Polystaltic pump, Buchler Instruments, Fort Lee, N.J.). The pump regulated the flow rate at about 10 ml/hr. Gum rubber tubing was used for the pumping action. The tubing had to be preconditioned for several hours by pumping solvents used in the fractionation. In this manner, most of the ultraviolet absorbing materials in the tubing were leached out before affecting the ultraviolet analysis of fractionated runs.

The effluent from the pump was directed to a trip reservoir in conjunction with an automatic turntable setup (G. M. Instrument Co., Greenville, Ill.). Five-milliliter fractions were collected in all fractionations.

Fractionation Technique

In the separation of diastereoisomer salt mixtures, distilled acetonitrile was chosen as the poor solvent to pack and charge the column. Mixtures of distilled water and 95% ethanol were used as good solvents. The reagent-grade ethanol was used without further purification.

The column, removed from the aluminum jacket for convenience, was packed with support as follows. A slurry of glass beads in acetoni-

trile was added to the column partially filled with acetonitrile. A wad of glass wool was placed at the base of the column to hold the support. The column was filled until the beads reached the corresponding level where the heating wire was located on the aluminum jacket. The packed column was gently hand tapped to insure complete settling of support.

The diastereoisomers were weighed and mixed together on a watch glass. The salts were pulverized with the end of a glass rod and mixed with a small quantity of glass beads. The mixture was transferred to the top of the column and distributed evenly on the support. At this point, only a slight excess of acetonitrile covered the glass beads. Another layer of glass beads, about 0.25 in., was added to the column to cover the mixture and to prevent disruption by the influent solvent. Following this, sufficient acetonitrile was added to cover the glass beads while thermal equilibrium on the column was established. This usually took a minimum of 1 hr. After this period of equilibrium, the solvent feed system was primed and the fractionation initiated.

The linear column temperature gradient, used for all fractionations, had a range of 52°C at the top to 12°C at the bottom of the glass column. The temperature conditions throughout the length of the column were verified by measurement with a copper–constantan thermocouple.

Preparation of Diastereoisomers

The salts were prepared by precipitation from a saturated solution of equal molar amounts of optically active compound and resolving agent. The stoichiometry of the salts was established by elemental analysis. When both the *d*- and *l*-forms of the compound were commercially available, the two diastereoisomers were prepared separately. The derivatives were then mixed into a 50:50 mixture or some other ratio and fractionated in the usual manner. Artificial mixing of the salts was helpful, when developing the fractionation procedure, to correlate the two resultant peaks with their respective salts. When the pure optical isomers were not available commercially, the diastereoisomers were prepared from an equal molar saturated solution of the racemic mixture and the resolving agent. Two crops of crystals were collected; the first crop was concentrated with respect to the less soluble salt, and the last crop was concentrated with respect to the more soluble salt. The crops were expected to be unequal mixtures of the two diastereoisomers. This preliminary partial separation of dia-

stereoisomers facilitated the correlation of fractionation peaks, particularly when the relative solubilities of the two salt derivatives were not known from the literature.

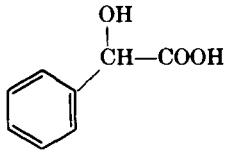
The radioactive malate and histidine diastereoisomers were prepared by precipitating the respective salts in a solution containing the radioactive components. About 2.5 μCi of *d*-histidine–carboxyl-^{14}C (New England Nuclear Corp., Boston, Mass.) were deposited in a 5-ml beaker. Twenty-five milligrams of a previously prepared *d*-histidine–*d*-tartrate salt were added followed by sufficient water to dissolve the mixture. The contents were warmed slightly to insure complete mixing, then evaporated to dryness in an evacuated dessicator. In a similar way, *l*-malic acid–4-^{14}C (Calbiochem, Los Angeles, Calif.) was used to prepare the corresponding *l*-malic acid–*d*-cinchonine salt.

Chromatographic Fractionation of Diastereoisomers

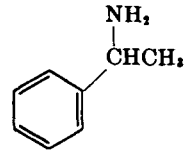
Acetonitrile was a satisfactory poor solvent for all the diastereoisomers investigated. The solvent is miscible with ethanol and water in all proportions and does not absorb in the near ultraviolet region of the spectrum.

The choice of solvent conditions in a fractionation run was mainly derived from experience in handling the samples. For instance, if a given pair of diastereoisomers was quite soluble in water or aqueous alcohol, the 500-ml mixing vessel was used instead of the 250-ml mixing vessel. This results in a smaller solvent concentration gradient. Using the same reasoning, when the salt pair was extremely soluble in pure water, aqueous alcohol was used as the good solvent. This approach allowed for a greater differential solubility between the diastereoisomers in the fractionation. Other workers (*21*,*22*) have used turbidimetric titrations to acquire information concerning the solvent system used in a fractionation.

A. Fractionation of the *d*-Mandelates (I) of *d*- and *l*-α-Phenylethylamine (II)



(I)



(II)

Racemic α-phenylethylamine (Matheson Coleman & Bell, East

Rutherford, N.J.) and mandelic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were used without further purification.

The 500-ml mixing vessel was used containing about 490 ml of acetonitrile as poor solvent and 10 ml of good solvent. The good solvent reservoir contained a solution of 40% distilled water in ethanol. The salt mixture sample sizes ranged from 20 to 80 mg, usually containing an equal amount of the two crops from the salt crystallization steps.

The column effluent was analyzed using ultraviolet absorption and polarimetric measurements. First, the ultraviolet absorption of the diastereoisomers in each tube fraction was measured at 257 mμ (Beckman Model DB recording spectrophotometer, Beckman Instruments, Fullerton, Calif.). The results were plotted as ultraviolet absorbance, corrected for any dilution, versus tube fraction.

Second, polarimetric measurements were used to confirm resolution. All the tube fractions that incorporated a single peak in the ultraviolet absorption analysis were combined. When separation was incomplete, two or more fractions between the two peaks were discarded to minimize cross-contamination. In order to collect sufficient salt from a corresponding peak to result in a good measurement of rotation, several fractionations were conducted. Each run was analyzed by ultraviolet absorption, and the fractions from the equivalent peaks were combined. This collection procedure was necessary since resolution of the salt mixture was impaired when sample fractionation sizes were too large. The combined fractions were allowed to evaporate to dryness. Approximately 100 mg of each diastereoisomer was collected.

At this point, the two salt samples, corresponding to the two peaks, were developed to regenerate the optically active base, α-phenylethylamine. The following procedure was used.

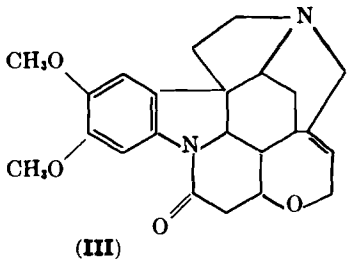
Each salt sample was dissolved in 1 ml of 0.1 *N* NaOH and added to a small test tube. To this solution, 1 ml of benzene was added. The mixture was then shaken and centrifuged. The benzene layer was transferred to another test tube with a medicine dropper. A similar procedure was repeated two more times on the original aqueous solution. The combined benzene portions were then treated with 0.5 ml of 0.1 *N* NaOH, shaken, and centrifuged. The separated benzene layer, containing the regenerated α-phenylethylamine, was then measured for optical rotation.

Separation of the two salts was confirmed when the two solutions gave measurable opposite rotations. To develop the extraction method,

40 mg of each of crop #1 and crop #2, from the preparative step, were regenerated separately as controls.

The optical rotation of the benzene solutions was measured using a 2-decimeter polarimeter tube of 3.8-ml capacity. The optical rotations were measured with a sodium lamp at room temperature.

B. Fractionation of *l*-Brucine (III) Diastereoisomers of *d*- and *l*-Tartaric Acid (IV)



(III)

$HO_2C—CHOHCHOH—CO_2H$

(IV)

In the fractionation runs, the 250-ml mixing vessel was used. The contents included 240 ml of acetonitrile and 10 ml of good solvent (50% solution of distilled water in ethanol). Sample sizes ranged from 3 to 60 mg. The ratio of the two diastereoisomers in the sample was variable by choice and was usually about 2:1.

The effluent tube fractions were analyzed by ultraviolet absorption and polarimetry. The brucine–tartrate salt concentration was analyzed by taking advantage of the high absorptivity of brucine in the ultraviolet spectral region at 303 mμ. Distilled water was used as a blank in the reference cell and also as a diluent of overly concentrated fractions for ultraviolet measurement. The absorbance of the tube fractions was plotted against the respective tube fractions.

After the development of the fractionation scheme, the separation was confirmed by polarimetry. The optically active tartaric acid was regenerated from the tartrate salt, extracted by ion exchange, and then measured for optical rotation. Because the concentration of tartaric acid in a typical tube fraction was low and its specific rotations also low ($[\alpha] = 14.5°$), several fractionations were conducted to collect sufficient sample. Large sample sizes in a fractionation were avoided so that the column would not be overloaded. About 100 mg of each diastereoisomer were collected from the corresponding ultraviolet absorbance peaks. Two or three tube fractions, separating the two peaks, were always discarded to decrease contamination. The combined test tube fractions were developed for measurement of optical rotation.

Because of the high solubility of tartaric acid in water, an ion-

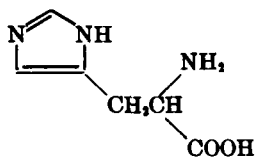
exchange technique was used to regenerate the acid rather than acidification and subsequent solvent extraction.

A small ion-exchange column was made from a disposable-type medicine dropper or a drawn out piece of 8-mm glass tubing. The glass column was partially filled with distilled water and an aqueous slurry of washed Dowex 50 ion-exchange resin (Dow Chemical Co., Midland, Mich.). The 100–200 mesh, strongly acidic resin had an exchange capacity of 5.1 meq/g dry weight. Only 0.5 g of the resin was necessary for the regeneration step.

The two tartrate samples from the fractionations were dissolved in a small quantity of water and added to two prepared ion-exchange columns. The solutions were eluted slowly, and the columns were then washed with approximately 20 ml of water. The total effluent, containing the optically active tartaric acid, was then measured for optical rotation. Approximately 100 mg each of the two tartrates, *d*-Al-B and *l*-Al-B, from the salt preparation steps were also regenerated and used as controls. The resultant rotations were compared with the rotations of the fractionated salts in order to estimate the recovery of the ion-exchange procedure.

The eluted solutions of optically active tartaric acid were evaporated by gentle heating to about 2 ml total volume. This volume was just sufficient to fill a 2-decimeter polarimeter tube.

C. Fractionation of the *d*-Tartrates of *d*- and *l*-Histidine (V)



(V)

The 250-ml mixing vessel was chosen for fractionation experiments. The contents included 240 ml of acetonitrile and 10 ml of the good solvent, distilled water. Sample fractionation sizes ranged from 2 to 25 mg.

Tube fractions containing the column effluent were analyzed for histidine concentration by the Pauly (*24,25*) colorimetric reagent and a radioactive tracer technique. The following includes a description of the Pauly reagent and the procedure for obtaining maximum color development for histidine.

Nine grams of sulfanilic acid was dissolved in 90 ml of concentrated HCl and diluted with 900 ml of distilled water (solution a).

A 5% solution of $NaNO_2$ and a 10% solution of Na_2CO_3 are indicated by solutions b and c, respectively. One part of solution a was mixed with one part of solution b and allowed to stand for a few minutes. Two parts of solution c were then added carefully because of effervescence.

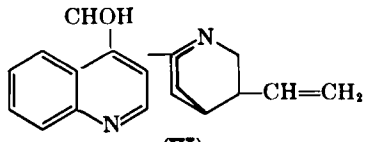
For color development, 0.5 ml was taken from each effluent tube fraction with a graduated 1-ml pipette and diluted with 2 to 5 ml of water. The dilution varied with color-intensity requirements and histidine concentration. To each diluted fraction, 1 ml of Pauly reagent was added to produce the color reaction. The spectrophotometric absorbance was measured at 495 mμ (Beckman Model DB spectrophotometer) and plotted versus the corresponding tube fraction. Blank fractions, which did not contain histidine, were colored pale yellow. One of these fractions was used as a reference for the spectrophotometric measurements.

The radioactive tracer method was developed using *d*-histidine-^{14}C and liquid scintillation counting techniques to aid in the location of *d*-histidine in the tube fractions.

The scintillation mixture* used to produce the phosphorescence of the beta-particle emission, had the following composition: 1.5 liters toluene (distilled), 0.5 liter Triton $\times$ 100 (Rohm & Haas Chemical Co., Philadelphia, Pa.), 0.250 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (Packard Instrument Co., Downers Grove, Ill.), and 11.0 g 2,5-diphenyloxazole (PPO) (Packard Instrument Co.).

Scintillation tubes (Packard Instrument Co.) were used as the sample holder for radiation measurement. Ten milliliters of the scintillation mixture was added to scintillation tubes containing 0.5 ml each of the respective effluent tube fractions (Packard Model 3310 Tri-Carb scintillation spectrophotometer). Radiation counts were run in triplicate, and averaged results (counts/min) were plotted versus the respective tube fraction.

D. Fractionation of *d*-Cinchonine (VI) Diastereoisomers of *d*- and *l*-Malic Acid (VII)



(VI)

$HO_2C—CHOHCH_2—CO_2H$

(VII)

* Courtesy of Dr. Carl L. Rollinson, University of Maryland.

The good solvent in the fractionations of *d*-A*d*-B and *l*-A*d*-B was a 3:1 mixture of ethanol and distilled water. The 250-ml mixing vessel was chosen and contained 240 ml of acetonitrile and 10 ml of good solvent. Sample fractionation sizes varied from 5 to 20 mg.

Column effluent fractions were analyzed by ultraviolet absorption (280 mμ) to follow total salt concentration, and by a radioactive tracer technique to distinguish between *d*- and *l*-malic acid. The general methods have been described above.

E. Fractionation of the *d*-Tartrates of *d*- and *l*-α-Phenylethylamine. The good solvent compositions ranged from pure ethanol to 70% ethanol in distilled water. Methanol was also tried as good solvent. The mixing vessel contained 240 ml of acetonitrile plus 10 ml of good solvent. Sample sizes ranged up to 40 mg in weight.

Column effluent analysis was accomplished by ultraviolet absorbance measurements with distilled water as the reference liquid. The absorbance was measured at 257 mμ, and the results were plotted versus the respective tube fractions.

RESULTS AND DISCUSSION

A. Separation of *d*-α-Phenylethylamine-*d*-Mandelic Acid and *l*-α-Phenylethylamine-*d*-Mandelic Acid. The first crop of crystals from the salt preparation was fractionated to obtain information on retention times and composition. The sample size was 50 mg. The resultant test tube fractions were diluted 1:1 with distilled water and analyzed by ultraviolet absorption (Fig. 2A). The plotted information shows two components with the major peak having a maximum absorbance at fraction #18.

A similar sample size from the second crop of crystals was fractionated using identical column conditions (Fig. 2B). The results indicate the presence of one major component occurring with a corresponding peak maximum at fraction #15.

The two plots gave complimentary information. Crop #1, the less soluble precipitate, was eluted from the column at a slower rate. A mixture of diastereoisomers was expected in one or both crops of crystals.

Following this preliminary investigation, fractionations were conducted using equal concentrations of the two crops. The results of fractionating 30 mg of crop #1 and 30 mg of crop #2 are illustrated in Fig. 3.

Experimental rotation data verified the salt compositions of the two

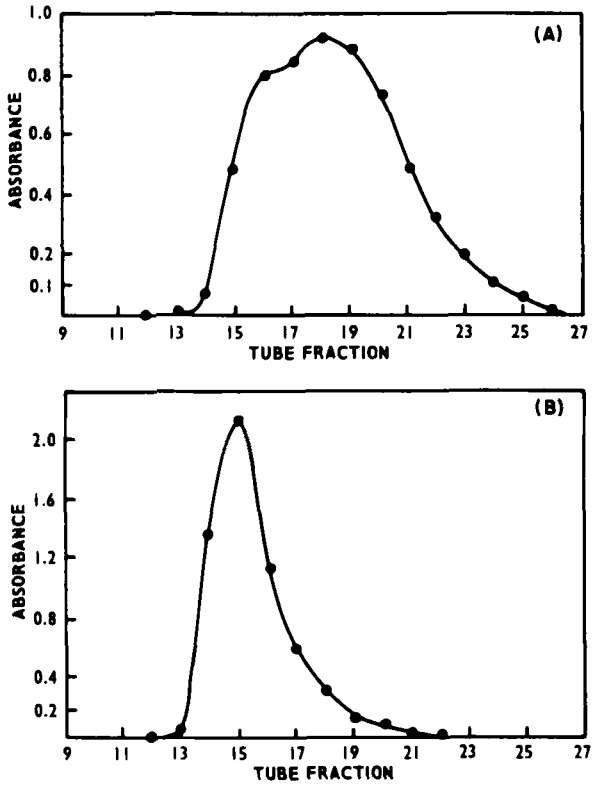


FIG. 2. Mixture fractionation of *d*-mandelates of *d*- and *l*-α-phenylethylamine. (A) Fractionation of crop #1; (B) fractionation of crop #2.

peaks. Approximately 100 mg each of *d*-B*d*-A and *l*-B*d*-A were collected from the fractions and developed to isolate the optically active base. The two controls indicated that the approximate recovery of the solvent extraction process was 60%. Calculations indicate that the expected rotation from 44 mg of pure base (corresponding to 100 mg of salt) would yield an absolute maximum rotation of 0.80°:

$$\begin{aligned} \alpha^\circ &= [\alpha]\, lc \\ &= 35\ (2)\ (1.15 \times 10^{-3}) \\ &= 0.80^\circ \end{aligned}$$

where

$$\begin{aligned} l &= 2 \text{ decimeters} \\ c &= \frac{0.044 \text{ g amine}}{3.8 \text{ ml polarimeter tube}} \\ [\alpha]_D &= 35^\circ \text{ in benzene} \end{aligned}$$

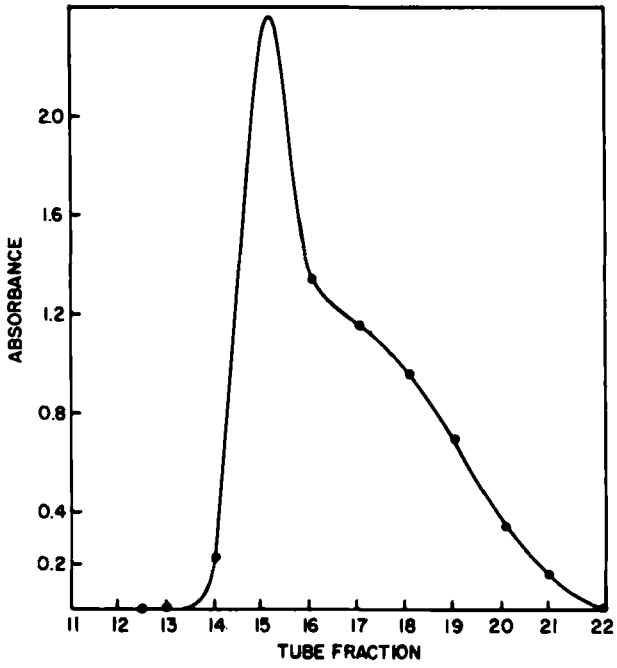


FIG. 3. Fractionation of *d*-mandelates of *d*- and *l*-α-phenylethylamine.

Assuming an analogous recovery to the controls (60%), the expected rotation is 0.48°. The average absolute rotation value obtained was 0.27°, indicating an estimated separation of almost 60%.

The rotation values confirmed the resolution. The two peaks plotted from absorbance measurements yielded opposite rotations with respect to the extracted optical isomers, *d*- and *l*-α-phenylethylamine.

B. Separation of *d*-Tartaric Acid-*l*-Brucine and *l*-Tartaric Acid-*l*-Brucine. The diastereoisomers were prepared in varied salt forms. Pasteur (*13*) prepared *l*-Al-*B* with 5 waters of hydration. The salt *d*-Al-B was not hydrated. On the other hand, Ladenburg and Fischl (*14*) crystallized *l*-Al-B with 14 waters of hydration, and *d*-Al-B was prepared with 5½ or 8 waters of hydration. Different temperatures and solvents affect the stoichiometry of the diastereoisomers.

These authors prepared the *d*-Al-B diastereoisomer with no water of hydration. However, elemental analysis of the *l*-Al-B salt indicated a complex salt form, containing several waters of hydration.

Each prepared diastereoisomer, *l*- and *d*-brucine tartrate, was fractionated individually to check purity and relative retention times. The

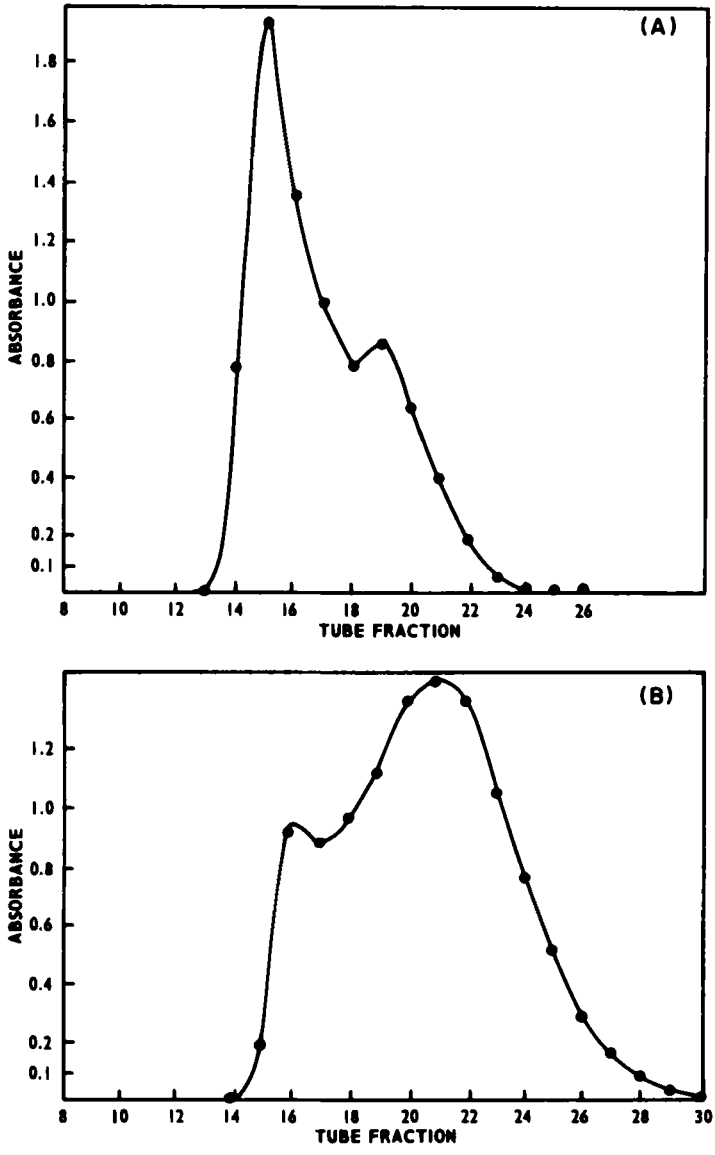


FIG. 4. Diastereoisomer fractionation of *d*- and *l*-tartrates of *l*-brucine. (A) Fractionation of *l*-brucine tartrate; (B) fractionation of *d*-brucine tartrate.

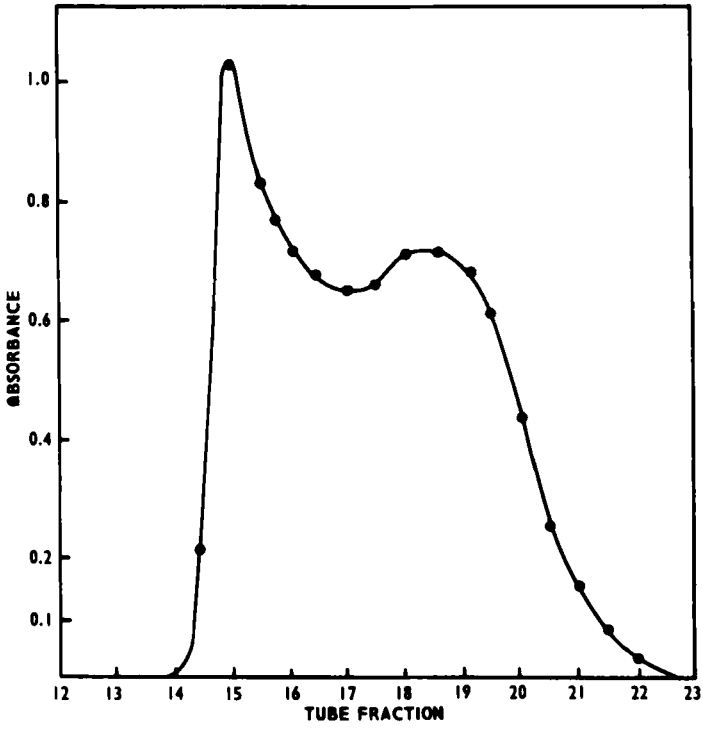


FIG. 5. Fractionation of *d*- and *l*-tartrates of *l*-brucine.

fractionation results of 3 mg of the *l*-brucine tartrate and 10 mg of the *d*-brucine tartrate are plotted in Fig. 4. The results indicate a distinct difference in column retention of the major respective peaks. It is also apparent that each salt contained a small amount of impurity. The minor peaks are attributed to either slight salt dissociation or to optical impurities in the commercial tartaric acid. Although each major peak was proven to be the respective brucine tartrate from absorbance and rotation data, some dissociation is not unlikely. Also, even though specific rotations of the respective tartaric acids were measured and agree generally with the diverse literature values, a 5–10% optical impurity is quite possible.

The peak symmetries resulting from fractionating a mixture of the two diastereoisomers were similar to those obtained from the resolution of racemic α-phenylethylamine. A mixture of *l*- and *d*-brucine tartrate corresponding to 1- and 2-mg weights, respectively, was fractionated (Fig. 5).

The ion-exchange method was appropriate for the regeneration and separation of tartaric acid from the combined diastereoisomer fractions. This technique was tested for recovery of tartaric acid by eluting weighed amounts of the *d*- and *l*-brucine tartrates. Average rotations of +0.30° and —0.49° were obtained from the regeneration of 95 mg of *d*-brucine tartrate and 125 mg of *l*-brucine tartrate, respectively. The average recovery compared to maximum was 75%. For best recovery, it was important to wash the ion-exchange column with a volume of water corresponding to 15 times the weight of ion-exchange resin used.

The combined samples of the collected fractionated salts included 71 mg of the *l*-brucine tartrate and 140 mg of the *d*-brucine tartrate. Using the ion-exchange recovery of 75%, the measured rotation results yielded an average separation of about 65%. Accordingly, the first peak formed from the fractionation of the tartrate salt mixtures is derived from *l*-tartaric acid, whereas the second peak is derived from *d*-tartaric acid.

C. Separation of *d*-Histidine–*d*-Tartaric Acid and *l*-Histidine–*d*-Tartaric Acid. Various attempts were unsuccessful in obtaining a measurable separation of the two diastereoisomeric salts, *l*-B*d*-A and *d*-B*d*-A. The results from a fractionation of an 11-mg mixture of the diastereoisomers are plotted in Fig. 6. The absorbance peak showed a slight shoulder on the ascending portion of the curve. Variations of the solvent conditions did not result in an improved separation.

The radioactive tracer method was ideally suited to measure a partial resolution. The results were specific and very sensitive (Fig. 6). The descending portion of the absorbance plot was, indeed, concentrated with respect to the spiked *d*-histidine-^{14}C–*d*-tartaric acid salt. The results are in agreement with solubility data given by Pyman (*18*). In the fractionation, *d*-B*d*-A was eluted from the column after *l*-B*d*-A, indicating that *d*-B*d*-A is less soluble in a more polar solvent than *l*-B*d*-A. Although the separation was partial, it is probable that the salt mixture could be separated using another solvent system.

D. Fractionation of *d*-Malic Acid–*d*-Cinchonine and *l*-Malic Acid–*d*-Cinchonine. These authors were unable to achieve a separation of the two malate diastereoisomers as was evident from ultraviolet effluent analysis. Data from the radioactive tracer technique revealed that salt decomposition was a fractionation problem.

Each prepared diastereoisomer, *d*-A*d*-B and *l*-A*d*-B, was fractionated individually to examine relative retention times. The two fractionations, conducted under identical column conditions, yielded single

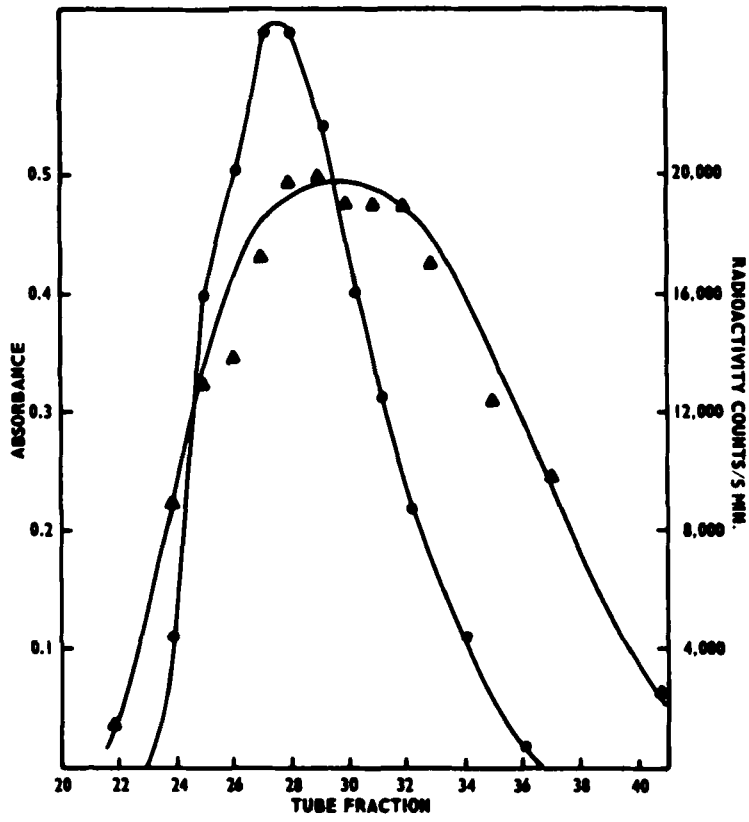


FIG. 6. Fractionation of *d*-tartrates of *d*- and *l*-histidine. Absorbance plot (circles); radioactivity plot (triangles).

peaks from ultraviolet effluent analysis. Comparing the locations of the respective peak maxima, indicated a small difference in retention times equivalent to two tube fractions.

As anticipated, a well-defined separation of a mixture of the two diastereoisomers was not obtained. Ten milligrams of each malate salt was mixed in the usual manner and fractionated under two solvent conditions. All other column conditions were held constant; one fractionation was conducted using a 250-ml mixing vessel, and the other fractionation employed the 500-ml mixing vessel. The use of the 250-ml mixing vessel resulted in better fractionation characteristics (less peak tailing); however, neither solvent gradient induced a separation.

Radioactive monitoring of the *l*-malic acid-^{14}C concentration showed

that the diastereoisomer decomposed under the respective column conditions. The ultraviolet absorbance and radioactivity data from the fractionation of a 20-mg equimolar mixture of the *d*- and *l*-malates are plotted in Fig. 7. Under normal conditions, the radioactive malic acid peak would be expected to correspond to the second ultraviolet absorbance peak. Dakin (*19*) reported that the *l*-malate diastereoisomer is less soluble than the corresponding *d*-malate diastereoisomer.

Attention was drawn to the fact that the ultraviolet absorbance peak symmetries differed from other fractionations using nonradioactive salts. The only factor that could account for this difference, which may be related to decomposition, was the preparation of the radioactive salt.

The preparation of radioactive *l*-malic acid–*d*-cinchonine was repeated under conditions similar to the preparation of the original inactive diastereoisomer. However, because of the small quantity of radioactive salt used in the preparation, the precipitated salt was col-

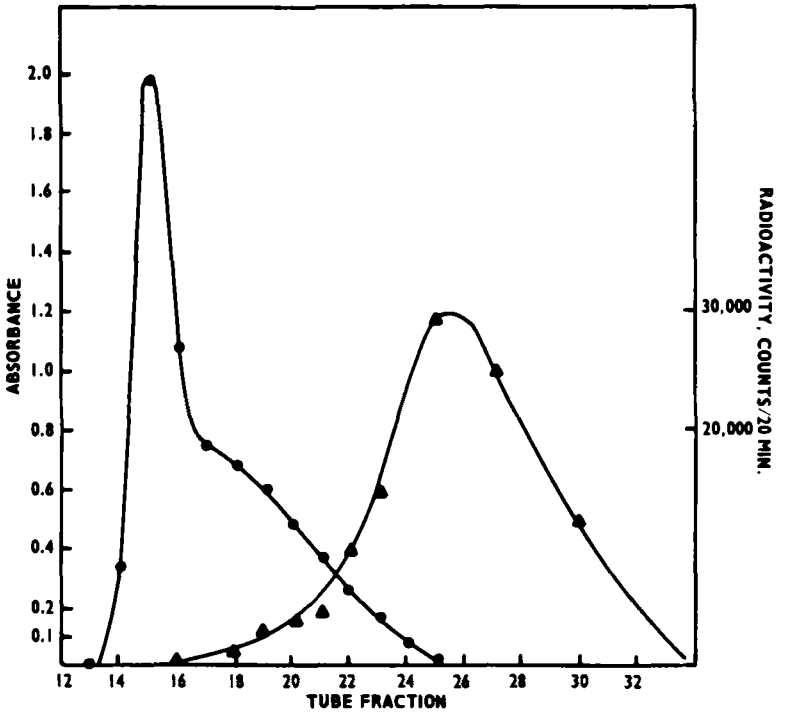


FIG. 7. Fractionation of *d*- and *l*-malates of cinchonine. Absorbance plot (circles); radioactivity plot (triangles).

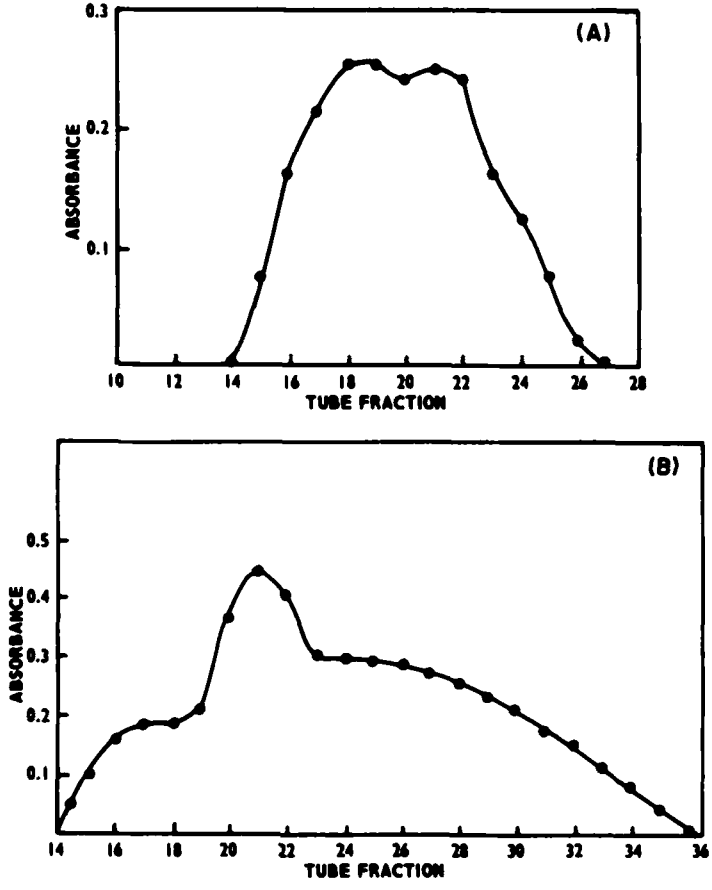


FIG. 8. Fractionation of *d*-tartrates of *d*- and *l*-α-phenylethylamine. (A) Good solvent, 70% ethanol in water; (B) good solvent, ethanol.

lected by complete evaporation of the solvent instead of by filtration from the solvent. The corresponding fractionation of the newly prepared salt again yielded two ultraviolet absorbance peaks of unequal size with the radioactive *l*-malic acid peak occurring later.

E. Fractionation of *d*-α-Phenylethylamine–*d*-Tartaric Acid and *l*-α-Phenylethylamine–*d*-Tartaric Acid. The fractionation results indicated that double salt formation occurred in the preparation of the diastereoisomers. The elemental analysis also indicated irregular salt

formation. The salt mixture contained α-phenylethylamine and tartaric acid in a ratio other than 1:1.

The salts were separated from a saturated solution in two crops in order to identify conveniently peaks in a fractionation. Fifteen grams of the first crop was fractionated, and the effluent tube fractions were analyzed by ultraviolet absorption (Fig. 8A). The fractionation of the more soluble crop of crystals (crop #2) resulted in one absorbance peak, whereas fractionation of the first crop, as can be seen, resulted in several peaks. The first crop of crystals was refractionated with a larger sample size (40 mg) and a less polar good solvent. Pure 95% ethanol was used as good solvent instead of 70% ethanol in distilled water (Fig. 8B). The presence of several absorbance peaks was more striking. Finally, 15 mg of the same crop was fractionated using a 75% solution of methanol in distilled water as good solvent. The resultant elution pattern was similar. Further investigation was not conducted on this salt system.

It was probable that more than one salt type crystallized from solution during the preparation of the salts. Decomposition of a normal salt on the column did not appear likely because of the number of peaks and the regularity of their sizes. The problem of double salt formation appears frequently in the literature of fractional crystallization. For example, the attempted resolution of pipecoline using *d*-tartaric acid was thwarted by irregular salt formation (*26*). As an illustration, *d*-B*d*-A and *l*-B*d*-A may be nicely crystalline salts of different solubility in a given solvent; however, resolution of *d*- and *l*-B by *d*-A often fails because of formation of the double salts, *d*-B*d*-A*l*-B*d*-A or 2*d*-A*l*-B*l*-A*l*-B. More complicated salts can form under varying conditions, and commonly hydrated salts form in aqueous solutions.

Chromatographic Fractionation Model

The multistage fractional precipitation mechanism proposed by Baker and Williams (*20*) has been refuted in many reports and defended in others. Many workers have noticed little or no difference in the degree of separation when no temperature gradient was imposed on the column. (*27*). In these cases, the successful separations were explained by a single-stage fractional elution model. In this interpretation, a separation is achieved by the fractional elution of the more

soluble components of the sample from the less soluble components due to the increasing gradient solvent power.

The literature does not allow a definite conclusion to be drawn concerning the mode of separation. In order for this to be possible, it is mandatory to standardize all separation parameters. These include flow rate, solvent gradient, preparation of sample, and sample size. The type of sample may also be an important consideration.

Several experiments were conducted to gain some insight into the mechanism of diastereoisomer separation. Fractionations were performed without a column temperature gradient to study the efficiency of fractional elution. The salt mixture of *d*-α-phenylethylamine-*d*-mandelic acid and *l*-α-phenylethylamine-*d*-mandelic acid was fractionated. The column was allowed to function at room temperature, and all other experimental conditions were similar to those previously described.

It was found that the fractionation without a temperature gradient does produce a separation of the diastereoisomers. However, the peaks are not as sharp or well defined as those peaks resulting from a column with an imposed temperature gradient. Nevertheless, the results do illustrate the important role that the fractional elution mechanism must play in chromatographic fractionation. A similar conclusion was reached from fractionations of *d*- and *l*-tartrate diastereoisomers of *l*-brucine using no column temperature gradient.

Depending on the type of sample, it would appear that a successful separation may be explained by a combination of both mechanisms. On the other hand, the column fractional elution mechanism may not be a simple single-stage process. As fresh solvent advances down the column, it mixes with poorer solvent in the interstitial column packing. This phenomenon could result in precipitation of the solute and subsequent redissolving when more fresh eluent advances. Furthermore, it is possible that the chromatographic fractionation method does not have as many fractionation stages as one would expect. The experimental time conditions may allow insufficient time for the equilibrium steps to occur during a run. Also, the continuous nature of gradient elution conditions could discourage the stepwise equilibrium conditions necessary for fractional precipitation and dissolving of a mixture on a column.

Many carefully controlled experiments using a variety of chemical compounds are required to bring clarification to this problem. It is hoped that the attention drawn by the mechanism discussion would

lead to some clarity. Perhaps a more thorough knowledge of the mode of separation will lead to alteration of column design and hence to further optimization of analytical conditions.

CONCLUSIONS

Two diastereoisomeric salt pairs were successfully fractionated by chromatographic fractionation, whereas partial resolution was achieved on a third pair. The *d*- and *l*-α-phenylethylamine diastereoisomers of *d*-mandelic acid and the *d*- and *l*-tartrates of *l*-brucine were separated by approximately 60%. The *d*- and *l*-histidine salts of *d*-tartaric acid were partially resolved.

Certain problems encountered in the development of the separation method were discussed. These included instances of diastereoisomer dissociation and irregular diastereoisomer formation. Also, several fractionations were conducted without an imposed column temperature gradient to gain some insight concerning the mode of separation. It seems likely that both column fractional elution and column fractional precipitation play contributing roles in a successful separation.

The chromatographic fractionation technique has two useful advantages over fractional crystallization for separating diastereoisomers. First, the handling of samples in the milligram range is easily done by column techniques, whereas larger sample sizes are required to obtain comparable results using fractional crystallization methods. Second, although both separation methods can be time-consuming, fractional crystallization is particularly tedious, whereas the developed column technique operates completely automatically.

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