REFERENCES

- Eckstein, H. C. (1927). *J. biol. Chem.* **73**, 363.
 Horn, D. H. S. & Hougen, F. W. (1953). *J. chem. Soc.* p. 3533.
 Idler, D. R. & Baumann, C. A. (1952). *J. biol. Chem.* **195**, 623.
 James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
 James, A. T. & Wheatley, V. R. (1956). *Biochem. J.* **63**, 269.
 Karnovsky, M. L. & Rapson, W. S. (1946). *J. Soc. chem. Ind., Lond.*, **65**, 138.
 MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1950). *J. invest. Derm.* **15**, 33.
 MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1952). *Biochem. J.* **52**, 161.
 Moore, P. R. & Baumann, C. A. (1952). *J. biol. Chem.* **195**, 615.
 Tiedt, J. & Truter, E. V. (1952). *J. chem. Soc.* p. 4628.
 Wheatley, V. R. (1953). *Biochem. J.* **53**, xxi.
 Wheatley, V. R. (1954). *Biochem. J.* **58**, 167.

The Separation of the C₆–C₁₂ Fatty Acids by Reversed-phase Partition Chromatography

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The monoterpene (C₁₀) acids are becoming of increasing interest to biochemists as possible intermediates in the biosynthesis of cholesterol, rubber and the carotenoids. The isolation and separation of small quantities of terpene acids should be facilitated by chromatography. Since these acids are all somewhat more polar than *n*-decanoic acid a chromatographic system capable of separating the straight-chain C₆–C₁₀ acids should be appropriate for their resolution.

Partition chromatography has been successfully applied to the separation of the C₈–C₁₀ fatty acids (Ramsey & Patterson, 1948; Peterson & Johnson, 1948; Boldingh, 1950; Nijkamp, 1951; Vandenhoevel & Hayes, 1952; Zbinovsky, 1955), and to the acids in the lower part of this range, C₆–C₈ (Moyle, Baldwin & Scarisbrick, 1948; Fairbairn & Harpur, 1951; Popják & Tietz, 1954). The lower fatty acids can also be separated by displacement analysis (Holman & Hagdahl, 1950). Gas-liquid chromatography affords an analytical separation with the highest resolution (James & Martin, 1952, 1956). Nevertheless, the convenience, high resolving power and larger capacity of reversed-phase partition chromatography as developed by Howard & Martin (1950) justified extending this method to include the C₆–C₁₂ acids.

Crombie, Comber & Boatman (1955) decreased the acetone concentration in the aqueous acetone-medicinal paraffin solvent system devised by Howard & Martin, and thereby extended the separations to include decanoic and octanoic acids. However, the aqueous methanol-chloroform system tentatively tested by Howard & Martin (1950) and developed by Wittenberg (1955) for another purpose

was found particularly well suited to the separation of the C₆–C₁₂ acids.

Columns of siliconized kieselguhr prepared with aqueous methanol-chloroform tend to lose the stationary phase during use (Howard & Martin, 1950). This difficulty may be obviated by the addition of a hydrocarbon to the chloroform phase (Bergström & Sjövall, 1951). Presumably the hydrocarbon achieves its effect by increasing the interfacial tension between the solvent phases (Howard & Martin, 1950). The stability of the column improves with increasing molecular weight of the added hydrocarbon. 2:2:4-Trimethylpentane stabilizes the columns far more than hexane or heptane, and decane affords a column which is stable to repeated use. Accordingly, a decane fraction, Skellysolve S, has been used to stabilize the columns described.

METHODS

Preparation of kieselguhr. Hyflo Super Cel (Johns-Manville Co. Ltd., New York, U.S.A.) was washed by decantation, twice with 3 vol. of 0.1N-HCl (Sjövall, 1953) and repeatedly with water until the supernatant fluid was relatively free of fine particles, which otherwise would leak from the columns (Silk & Hahn, 1954). The washed kieselguhr was dried at 110°, and, when cool, stirred while passing through it a stream of dry nitrogen previously drawn through liquid dichlorodimethylsilane. The treated kieselguhr was washed with methanol, until the washings were no longer acid, was dried at 110° and stored for use.

Solvents. Solvents were used without special purification. Skellysolve S (boiling range 150–202°) is stated by the manufacturer (Skelly Oil Co., Kansas City, Missouri, U.S.A.) to contain some nonanes and undecanes and a considerable quantity of *n*- and *iso*-decanes.

Columns. The columns used were 1.8 cm. in diameter. Treated kieselguhr (15 g.) and the less polar solvent phase (13 ml.) were stirred together until a homogeneous powder was obtained. This was suspended in the more polar phase and packed into the column with the aid of a perforated stainless-steel plunger as described by Howard & Martin (1950). The column obtained was 11 cm. long. The solvent was run down the column at the rate of 30 ml./hr., and collected in 3 ml. fractions. Columns may be used repeatedly but must be protected from fluctuations in room temperature which would disturb the mutual solubilities of the solvent phases.

Loading. The acids to be resolved were added to the column in solution in the more polar solvent phase containing not more than 15 mg. of acids/ml. A total of about 0.2 m-mole of acids may be separated, although the capacity for dodecanoic acid is somewhat more limited. The quantity of acids which may be separated is limited by solvent-solute interactions, which cause an increase in the relative volume of the less polar phase.

Titration. The effluent fractions were titrated with methanolic 0.1 N-NaOH, delivered from a microburette, to the end-point of phenol red.

Solvent system. Of the several tried, only two solvent systems were found useful. The mixture chloroform-Skellysolve S-water-methanol (8:1.5:12:20, by vol.), served to separate acids up to C₁₀, as exemplified in Figs. 1 and 3. The mixture of the same components in the proportion

8:1.75:11:20 (by vol.) served to extend the separation to include dodecanoic acid (Fig. 2). Either solvent mixture may be used to separate geranic acid from other acids (Fig. 3). The solvents were prepared by mixing the four components, allowing the mixture to cool, and separately filtering the two phases through dry filter paper before use. The less polar, chloroform-rich, lower phase served as the stationary phase; the more polar, aqueous methanol, upper phase served as the moving phase of the column.

RESULTS AND DISCUSSION

The separation of *n*-hexanoic, *n*-octanoic, *n*-decanoic and dodecanoic acids is illustrated in Figs. 1 and 2. *n*-Valeric and *n*-hexanoic acids are partially resolved; *n*-butyric acid moves close to the solvent front. The recoveries are about 90 %.

The terpene acid, geranic acid, is separated from decanoic acid with each of the solvent mixtures described (Fig. 3). The mono- and di-hydroxy terpene acids should travel down the column more rapidly than geranic acid and will be well separated from geranic acid and from each other, since other compounds differing only in the number of their hydroxyl groups are widely separated on chromatograms of this type (Howard & Martin, 1950;

Fig. 1. Separation of a mixture of *n*-hexanoic, *n*-octanoic and *n*-decanoic acids (75 μ moles of each) on a column (1.8 cm. $\times$ 12.4 cm.). Solvent system: chloroform-Skellysolve S-water-methanol (8:1.5:12:20, by vol.).

Fig. 2. Separation of a mixture of *n*-octanoic ($75\ \mu\text{moles}$), *n*-decanoic ($75\ \mu\text{moles}$) and *n*-dodecanoic acids ($61\ \mu\text{moles}$) on column ($1.8\ \text{cm.} \times 11.7\ \text{cm.}$). Solvent system: chloroform-Skellysolve S-water-methanol ($8:1.75:11:20$, by vol.).

Fig. 3. Separation of mixtures of geranic and *n*-decanoic acids ($75\ \mu\text{moles}$ of each) on columns ($1.8\ \text{cm.} \times 12.3\ \text{cm.}$).
○, Solvent system as Fig. 1; ●, solvent system as Fig. 2.

Wittenberg, 1955). No terpene acids other than geranic acid were available for testing. However, the position of the peak for any acid may be predicted from an experimentally determined value of the distribution coefficient (α), since the chromatogram described behaves as a theoretical partition chromatogram (Martin & Synge, 1941). The pertinent values necessary for the calculation are: cross-sectional area of column (A), 255 mm.²; cross-sectional area of non-mobile phase (A_s), 85 mm.²; cross-sectional area of mobile phase (A_L), 113 mm.².

It is of interest that columns similar to those described here have been useful for the separation of three other groups of acidic lipids, the glycerol phosphatides (Garvin & Karnovsky, 1954), the bile acids (Bergström & Sjövall, 1951; Sjövall, 1953), and *N*-succinylsphingosine and its congeners (Wittenberg, 1955).

SUMMARY

1. The method of reversed-phase partition chromatography has been extended to the separation of the C₆-C₁₂ fatty acids.

2. This type of chromatography is applicable to the separation of the terpene acids.

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REFERENCES

- Bergström, S. & Sjövall, J. (1951). *Acta chem. scand.* **5**, 1267.
 Boldingh, J. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 247.
 Crombie, W. M. L., Comber, R. & Boatman, S. G. (1955). *Biochem. J.* **59**, 309.
 Fairbairn, D. & Harpur, R. P. (1951). *Canad. chem. J.* **29**, 633.
 Garvin, J. E. & Karnovsky, M. (1954). *Fed. Proc.* **13**, 215.
 Holman, R. T. & Hagdahl, L. (1950). *J. biol. Chem.* **182**, 421.
 Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
 James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
 James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
 Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
 Moyle, V., Baldwin, E. & Scarisbrick, R. (1948). *Biochem. J.* **43**, 308.
 Nijkamp, H. J. (1951). *Analyt. chim. acta*, **5**, 325.
 Peterson, M. H. & Johnson, M. J. (1948). *J. biol. Chem.* **174**, 775.
 Popják, G. & Tietz, A. (1954). *Biochem. J.* **56**, 46.
 Ramsey, L. L. & Patterson, W. I. (1948). *J. Ass. off. agric. Chem., Wash.*, **31**, 139.
 Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **56**, 406.
 Sjövall, J. (1953). *Acta physiol. scand.* **29**, 232.
 Vandenheuvel, F. A. & Hayes, E. R. (1952). *Analyt. Chem.* **24**, 960.
 Wittenberg, J. (1955). *J. biol. Chem.* **216**, 379.
 Zbinovsky, V. (1955). *Analyt. Chem.* **27**, 764.

The Synthesis of some Analogues of Glutathione

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Several analogues of glutathione were required for studies on glyoxalase described in a later paper. The preparation of these compounds was undertaken by synthesis from amino acids and by modification of glutathione itself.

King & Kidd (1949, 1951) and King, Jackson & Kidd (1951) have shown that when phthalylglutamic anhydride is condensed with amino acids or peptides γ - and not α -glutamyl derivatives are obtained, so this route was first investigated.

Phthalyl-DL-glutamic anhydride was allowed to react with glycylglycine in acetic acid, giving

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phthalyl- γ -DL-glutamylglycylglycine. The phthalyl group was then removed with phenylhydrazine (Boissonnas & Schumann, 1952) and, after recrystallization, the resulting γ -DL-glutamylglycylglycine behaved as a pure compound on paper chromatograms. When this compound was heated with ninhydrin, one mole of carbon dioxide was evolved/mole of peptide; since only free carboxyl groups with adjacent free amino groups react in this way, the peptide was evidently γ - not α -glutamylglycylglycine.

In a similar manner, phthalyl- γ -DL-glutamyl-DL-alanyl-glycine was obtained from DL-alanyl-glycine, and the removal of the phthalyl group gave γ -DL-glutamyl-DL-alanyl-glycine. To confirm the identity